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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 15/10, 33/50, 15/14 — A2

(11) International Publication Number: **WO 97/14028**

(43) International Publication Date: 17 April 1997 (17.04.97)

(21) International Application Number: PCT/US96/16198

(22) International Filing Date: 10 October 1996 (10.10.96)

(30) Priority Data:
08/540,814 — 11 October 1995 (11.10.95) — US
08/542,401 — 11 October 1995 (11.10.95) — US

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(81) Designated States: AL, AM, AT, AU, BA, BB, BG, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: MULTIPLEXED ANALYSIS OF CLINICAL SPECIMENS APPARATUS AND METHOD

(57) Abstract

A method for the multiplexed diagnostic and genetic analysis of enzymes, DNA fragments, antibodies, and other biomolecules comprises the steps of constructing an appropriately labeled beadset, exposing the beadset to a clinical sample, and analyzing the combined sample/beadset by flow cytometry is disclosed. Flow cytometric measurements are used to classify, in real-time, beads within an exposed beadset and textual explanations, based on the accumulated data obtained during real-time analysis, are generated for the user. The inventive technology enables the simultaneous, and automated, detection and interpretation of multiple biomolecules or DNA sequences in real-time while also reducing the cost of performing diagnostic and genetic assays.

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MULTIPLEXED ANALYSIS OF CLINICAL SPECIMENS APPARATUS AND METHOD

Microfiche appendix A contains a listing of selected Visual Basic and C programming source code in accordance with the inventive multiplexed assay method. Microfiche appendix A, comprising 1 sheet having a total of 58 frames, contains material which is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent disclosure, as it appears in the Patent and Trademark Office patent files or records, but otherwise reserves all copyright rights whatsoever.

The invention relates generally to laboratory diagnostic and genetic analysis and, more particularly, to a flow cytometric method for the simultaneous and multiplexed diagnostic and genetic analysis of clinical specimens.

Analysis of clinical specimens is important in science and medicine. A wide variety of assays to determine qualitative and/or quantitative characteristics of a specimen are known in the art. Detection of multiple analytes, or separately identifiable characteristics of one or more analytes, through single-step assay processes are presently not possible or, to the extent possible, have provided only very limited capability and have not yielded satisfactory results. Some of the reasons for these disappointing results include the extended times typically required to enable the detection and classification of multiple analytes, the inherent limitations of known reagents, the low sensitivities achievable in prior art assays which often lead to significant analytical errors and the unwieldy collection, classification, and analysis of prior art algorithms vis à vis the large amounts of data obtained and the subsequent computational requirements to analyze that data.

Clearly, it would be an improvement in the art to have adequate apparatus and methods for reliably performing real-time multiple determinations, substantially simultaneously, through a single or limited step assay process. A capability to perform simultaneous, multiple

determinations in a single assay process is known as "multiplexing" and a process to implement such a capability is a "multiplexed assay."

Flow Cytometry

One well known prior art technique used in assay procedures for which a multiplexed assay capability would be particularly advantageous is flow cytometry. Flow cytometry is an optical technique that analyzes particular particles in a fluid mixture based on the particles' optical characteristics using an instrument known as a flow cytometer. Background information on flow cytometry may be found in Shapiro, "Practical Flow Cytometry," Third Ed. (Alan R. Liss, Inc. 1995); and Melamed et al., "Flow Cytometry and Sorting," Second Ed. (Wiley-Liss 1990), which are incorporated herein by reference.

Flow cytometers hydrodynamically focus a fluid suspension of particles into a thin stream so that the particles flow down the stream in substantially single file and pass through an examination zone. A focused light beam, such as a laser beam illuminates the particles as they flow through the examination zone. Optical detectors within the flow cytometer measure certain characteristics of the light as it interacts with the particles. Commonly used flow cytometers such as the Becton-Dickinson Immunocytometry Systems "FACSCAN" (San Jose, CA) can measure forward light scatter (generally correlated with the refractive index and size of the particle being illuminated), side light scatter (generally correlated with the particle's size), and particle fluorescence at one or more wavelengths. (Fluorescence is typically imparted by incorporating, or attaching a fluorochrome within the particle.) Flow cytometers and various techniques for their use are described in, generally, in "Practical Flow Cytometry" by Howard M. Shapiro (Alan R. Liss, Inc., 1985) and "Flow Cytometry and Sorting, Second Edition" edited by Melamed et al. (Wiley-Liss, 1990).

One skilled in the art will recognize that one type of "particle" analyzed by a flow cytometer may be man-made microspheres or beads. Microspheres or beads for use in flow cytometry are generally known in the art and may be obtained from manufacturers such as Spherotech (Libertyville, IL), and Molecular Probes (Eugene, OR).

Although a multiplexed analysis capability theoretically would provide enormous benefits in the art of flow cytometry, very little multiplexing capability has been previously achieved. Prior multiplexed assays have obtained only a limited number of determinations. A review of some of these prior art techniques is provided by McHugh, "Flow Microsphere Immunoassay for the Quantitative and Simultaneous Detection of Multiple Soluble Analytes," in Methods in Cell Biology, 42, Part B, (Academic Press, 1994). For example, McHugh et al., "Microsphere-Based Fluorescence Immunoassays Using Flow Cytometry Instrumentation," in Clinical Flow Cytometry Ed. K.D. Bauer, et al., Williams and Williams, Baltimore, MD, 1993, 535-544, describe an assay where microspheres of different sizes are used as supports and the identification of microspheres associated with different analytes was based on distinguishing a microsphere's size. Other references in this area include Lindmo, et al., "Immunometric Assay by Flow Cytometry Using Mixtures of Two Particle Types of Different Affinity," J. Immun. Meth., 126, 183-189 (1990); McHugh, "Flow Cytometry and the Application of Microsphere-Based Fluorescence Immunoassays," Immunochemica, 5, 116 (1991); Horan et al., "Fluid Phase Particle Fluorescence Analysis: Rheumatoid Factor Specificity Evaluated by Laser Flow Cytophotometry" in Immunoassays in the Clinical Laboratory, 185-198 (Liss 1979); Wilson et al., "A New Microsphere-Based Immunofluorescence Assay Using Flow Cytometry," J. Immunological Methods, 107, 225-230 (1988); and Fulwyler et al., "Flow Microsphere Immunoassay for the Quantitative and Simultaneous Detection of Multiple Soluble Analytes," Meth. Cell Biol., 33, 613-629 (1990).

The above cited methods have been unsatisfactory as applied to provide a fully multiplexed assay capable of real-time analysis of more than a few different analytes. For example, certain of the assay methods replaced a single ELISA procedure with a flow cytometer-based assay. These methods were based on only a few characteristics of the particles under analysis and enabled simultaneous determination of only a very few analytes in the assay. Also, the analytic determinations made were hampered due to software limitations including the inability to perform real-time processing of the acquired assay data. In summary, although it has been previously hypothesized that flow cytometry may possibly be adapted to operate and

provide benefit in a multiple analyte assay process, such an adaptation has not in reality been accomplished.

Analysis of Genetic Information

The availability of genetic information and association of disease with mutation(s) of critical genes has generated a rich field of clinical analysis. In particular, the use polymerase chain reaction (PCR) and its variants have facilitated genetic analysis. A major advance in this field is described in our co-pending and contemporaneously filed U.S. Application entitled "Methods and Compositions for Flow Cytometric Determination of DNA Sequences." This co-pending application describes a powerful flow cytometric assay for PCR products, which may be multiplexed in accordance with the present invention. A multiplexed flow cytometric assay for PCR reaction products would provide a significant advantage in the field of genetic analysis.

Recent advances in genetic analyses have provided a wealth of information regarding specific mutations occurring in particular genes in given disease states. Consequently, use of an individual's genetic information in diagnosis of disease is becoming increasingly prevalent. Genes responsible for disease have been cloned and characterized in a number of cases, and it has been shown that responsible genetic defects may be a gross gene alteration, a small gene alteration, or even in some cases, a point mutation. There are a number of reported examples of diseases caused by genetic mutations. Testing of gene expression by analysis of cDNA or mRNA, and testing of normal genes and alleles, as in cases of tissue typing and forensics, are becoming widespread. Other uses of DNA analysis, for example in paternity testing, etc., are also important and can be used in accordance with the invention.

Current techniques for genetic analysis have been greatly facilitated by the development and use of polymerase chain reaction (PCR) to amplify selected segments of DNA. The power and sensitivity of the PCR has prompted its application to a wide variety of analytical problems in which detection of DNA or RNA sequences is required.

PCR is capable of amplifying short fragments of DNA, providing short (20 bases or more) nucleotides are supplied as primers. The primers anneal to either end of a span of denatured DNA target and, upon renaturation, enzymes synthesize the intervening complementary sequences by extending the primer along the target strand. During denaturation, the temperature is raised to break apart the target and newly synthesized complementary sequence. Upon cooling, renaturation and annealing, primers bind to the target and the newly made opposite strand and now the primer is extended again creating the complement. The result is that in each cycle of heating and renaturation followed by primer extension, the amount of target sequence is doubled.

One major difficulty with adoption of PCR is the cumbersome nature of the methods of analyzing the reaction's amplified DNA products. Methods for detecting genetic abnormalities and PCR products have been described but they are cumbersome and time consuming. For example, U.S. Patent No. 5,429,923 issued July 4, 1995 to Seidman, et al., describes a method for detecting mutations in persons having, or suspected of having, hypertrophic cardiomyopathy. That method involves amplifying a DNA sequence suspected of containing the disease associated mutation, combining the amplified product with an RNA probe to produce an RNA-DNA hybrid and detecting the mutation by digesting unhybridized portions of the RNA strand by treating the hybridized product with an RNAse to detect mutations, and then measuring the size of the products of the RNAse reaction to determine whether cleavage of the RNA molecule has occurred.

Other methods used for detecting mutations in DNA sequences, including direct sequencing methods (Maxim and Gilbert, Proc. Natl. Acad. Sci. U.S.A., 74, 560-564, 1977); PCR amplification of specific alleles, PASA (Botttema and Sommer, Muta. Res., 288, 93-102, 1993); and reverse dot blot method (Kawasaki, et al., Methods in Enzymology, 218, 369-81, 1993) have been described. These techniques, while useful, are time consuming and cumbersome and for that reason are not readily adaptable to diagnostic assays for use on a large scale.

At least one use of flow cytometry for the assay of a PCR product has been reported but that assay has not been adapted to multiplexing. See Vlieger et al., "Quantitation of Polymerase Chain Reaction Products by Hybridization-Based Assays with Fluorescent Colorimetric, or

Chemiluminescent Detection," Anal. Biochem., 205, 1-7 (1992). In Vlieger et al. a PCR product was labeled using primers that contained biotinylated nucleotides. Unreacted primers were first removed and the amplified portion annealed with a labeled complementary probe in solution. Beaded microspheres of avidin were then attached to the annealed complementary material. The avidin beads bearing the annealed complementary material were then processed by a flow cytometer. The procedure was limited, inter alia, in that avidin beads having only a single specificity were employed. Further, real-time analysis of the assay's data was not possible.

Data Manipulation

The large volume of data typically generated during flow cytometric multiple analyte assays, combined with the limited capabilities of prior techniques to collect, sort and analyze such data have provided significant obstacles in achieving a satisfactory multiplexed assay. The computing methods used in prior art flow cytometric analyses have generally been insufficient and unsuited for accurately and timely analyzing large volumes of data such as would be generated by multiplexed assays; particularly when more than two analytes (or properties of a single analyte) are to be simultaneously determined.

The present invention enables the simultaneous determination of multiple distinct analytes to a far greater degree than existing techniques. Further, the invention provides an improved data classification and analysis methodology that enables the meaningful analysis of highly multiplexed assays in real-time. The invention is broadly applicable to multiplexed analysis of a number of analytes in a host of bioassays in which there is currently a need in the art.

The present invention provides improved methods, instrumentation, and products for detecting multiple analytes in a fluid sample by flow cytometric analysis and for analyzing and presenting the data in real-time. An advantage of the invention is that it allows one rapidly and simultaneously to detect a wide variety of analytes of interest in a single assay step.

The invention employs a pool of bead subsets. The individual subsets are prepared so that beads within a subset are relatively homogeneous but differ in at least one distinguishing characteristic from beads in any other subset. Therefore, the subset to which a bead belongs can readily be determined after beads from different subsets are pooled.

In a preferred embodiment, the beads within each subset are uniform with respect to at least three and preferably four known classification parameter values measured with a flow cytometer: e.g., forward light scatter (C_1) which generally correlates with size and refractive index; side light scatter (C_2) which generally correlates with size; and fluorescent emission in at least one wavelength (C_3), and preferably in two wavelengths (C_3 and C_4), which generally results from the presence of fluorochrome(s) in or on the beads. Because beads from different subsets differ in at least one of the above listed classification parameters, and the classification parameters for each subset are known, a bead's subset identity can be verified during flow cytometric analysis of the pool in a single assay step and in real-time.

Prior to pooling subsets of beads to form a beadset, the beads within each subset can be coupled to a reactant that will specifically react with a given analyte of interest in a fluid sample to be tested. Usually, different subsets will be coupled to different reactants so as to detect different analytes. For example, subset 1 may be labeled so as to detect analyte A (AnA); subset 2 may be labeled so as to detect analyte B (AnB); etc.

At some point prior to assay, the variously labeled subsets are pooled. The pooled beads, or beadset, are then mixed with a fluid sample to test for analytes reactive with the various reactants bound to the beads. The system is designed so that reactions between the reactants on the bead surfaces and the corresponding analytes in the fluid sample will cause changes in the intensity of at least one additional fluorescent signal (F_m) emitted from a fluorochrome that fluoresces at a wavelength distinct from the wavelengths of classification parameters C_3 or C_4. The F_m signal serves as a "measurement signal," that is, it indicates the extent to which the reactant on a given bead has undergone a reaction with its corresponding analyte. The F_m signal may result from the addition to the assay mixture of fluorescently labeled "secondary" reagent that binds to the bead surface at the site where a reactant-analyte reaction has occurred.

When the mixture (pooled beads and fluid sample) is run through a flow cytometer, each bead is individually examined. The classification parameters, e.g., C_1, C_2, C_3, and C_4, are measured and used to classify each bead into the subset to which it belongs and, therefore, identify the analyte that the bead is designed to detect. The F_m value of the bead is determined to indicate the concentration of analyte of interest in the fluid sample. Not only are many beads from each subset rapidly evaluated in a single run, multiple subsets are evaluated in a single run. Thus, in a single-pass and in real-time a sample is evaluated for multiple analytes. Measured F_m values for all beads assayed and classified as belonging to a given subset may be averaged or otherwise manipulated statistically to give a single meaningful data point, displayed in histogram format to provide information about the distribution of F_m values within the subset, or analyzed as a function of time to provide information about the rate of a reaction involving that analyte.

In a preferred embodiment, the beads will have two or more fluorochromes incorporated within or on them so that each of the beads in a given subset will possess at least four different classification parameters, e.g., C_1, C_2, C_3, and C_4. For example, the beads may be made to contain a red fluorochrome (C_3), such as nile red, and bear an orange fluorochrome (C_4), such as Cy3 or phycoerythrin. A third fluorochrome, such as fluorescein, may be used as a source of the C_n or F_m signal. As those of skill in the art will recognize, additional fluorochromes may be used to generate additional C_n signals. That is, given suitable fluorochromes and equipment, those of skill in the art may use multiple fluorochromes to measure a variety of C_n or F_m values, thus expanding the multiplexing power of the system even further.

In certain applications designed for more quantitative analysis of analyte concentrations or for kinetic studies, multiple subsets of beads may be coupled to the same reactant but at varying concentrations so as to produce subsets of beads varying in density of bound reactant rather than in the type of reactant. In such an embodiment, the reactant associated with classification parameter C_4, for example, may be incorporated directly into the reactive reagent

that is coupled to the beads, thereby allowing C_4 conveniently to serve as an indicator of density of reactant on the bead surface as well as an indicator of reactant identity.

To prepare subsets varying in reactant density one may, for example, select, isolate, or prepare a starting panel of different subsets of beads, each subset differing from the other subsets in one or more of C_1, C_2, or C_3. Each of those subsets may be further subdivided into a number of aliquots. Beads in each aliquot may be coupled with a reactant of choice that has been fluorescently labeled with a fluorochrome associated with C_4 (e.g., Analyte A labeled with Cy3) under conditions such that the concentration or density of reactant bound to the beads of each aliquot will differ from that of each other aliquot in the subset. Alternatively, an entire subset may be treated with the C_4 fluorochrome under conditions that produce a heterogeneous distribution of C_4 reactant on beads within the subset. The subset may then be sorted with a cell sorter on the basis of the intensity of C_4 to yield further subsets that differ from one another in C_4 intensity.

One limitation of the alternative embodiment of using C_4 labeled reactant as a classification agent is that one must design the system so that the value of C_4 as a classification parameter is not lost. Therefore, one must take care to assure that the C_4 intensities of all subsets carrying reagent A differs from the C_4 intensities of all subsets carrying reagents B, C, and so forth. Otherwise, C_4 would not be useful as a parameter to discriminate reactant A from reactant B, etc.

With either embodiment, the number of subsets that can be prepared and used in practice of the invention is theoretically quite high, but in practice will depend, *inter alia*, on the level of homogeneity within a subset and the precision of the measurements that are obtained with a flow cytometer. The intra-subset heterogeneity for a given parameter, e.g., forward angle light scatter C_1, correlates inversely with the number of different subsets for that parameter that can be discriminated by flow cytometric assay. It is therefore desirable to prepare subsets so that the coefficients of variation for the value of each classification parameter (C_1, C_2, C_3, and C_4) to be used in a given analysis is minimized. Doing this will maximize the number of subsets that can

be discriminated by the flow cytometer. Bead subsets may be subjected to flow cytometric sorting or other procedures at various different points in preparation or maintenance of the bead subsets to increase homogeneity within the subset. Of course, with simple assays designed to detect only a few different analytes, more heterogeneity can be allowed within a subset without compromising the reliability of the assay.

In an illustrative embodiment set forth here to explain one manner in which the invention can work in practice, the beads are used to test for a variety of antibodies in a fluid sample. A panel of bead subsets having known varying C_1, C_2, C_3, and C_4 values is first prepared or otherwise obtained. The beads within each subset are then coupled to a given antigen of interest. Each subset receives a different antigen. The subsets are then pooled to form an assay beadset and may be stored for later use and/or sold as a commercial test kit.

In the assay procedure, the beads are mixed with the fluid to be analyzed for antibodies reactive with the variety of antigens carried on the beads under conditions that will permit antigen-antibody interaction. The beads are labeled with a "secondary" reagent that binds to antibodies bound to the antigens on the beads and that also bears the measurement fluorochrome associated with parameter F_m (e.g., fluorescein). A fluoresceinated antibody specific for immunoglobulin may be used for this purpose. The beads are then run through a flow cytometer, and each bead is classified by its characteristic classification parameters as belonging to subset-1, subset-2, etc. At the same time, the presence of antibodies specific for antigen A, B, etc., can be detected by measuring green fluorescence, F_m, of each bead. The classification parameters C_1, C_2, C_3, and C_4 allow one to determine the subset to which a bead belongs, which serves as an identifier for the antigen carried on the bead. The F_m value of the bead indicates the extent to which the antibody reactive with that antigen is present in the sample.

Although assays for antibodies were used above as an illustration, those of ordinary skill in the art will recognize that the invention is not so limited in scope, but is widely applicable to detecting any of a number of analytes in a sample of interest. For example, the methods described here may be used to detect enzymes or DNA or virtually any analyte detectable by virtue of a given physical or chemical reaction. A number of suitable assay procedures for

detection and quantification of enzymes and DNA (particularly as the result of a PCR process) are described in more detail below.

The present invention also provides a significant advance in the art by providing a rapid and sensitive flow cytometric assay for analysis of genetic sequences that is widely applicable to detection of RNA, differing alleles, and any of a number of genetic abnormalities. In general, the methods of the present invention employ a competitive hybridization assay using DNA coupled microspheres and fluorescent DNA probes. Probes and microsphere-linked oligonucleotides could also include RNA, PNA, and non-natural nucleotide analogs.

In practice of the invention, oligonucleotides from a region of a gene of interest, often a polymorphic allele or a region to which a disease associated mutation has been mapped, are synthesized and coupled to a microsphere (bead) by standard techniques such as by carbodiimide coupling. A fluorescent oligonucleotide, complementary to the oligonucleotide on the bead, is also synthesized. To perform a test in accordance with the invention, DNA which is to be tested is purified and either assayed unamplified, or subjected to amplification by PCR, RT-PCR, or LCR amplification using standard techniques and PCR initiation probes directed to amplify the particular region of DNA of interest. The PCR product is then incubated with the beads under conditions sufficient to allow hybridization between the amplified DNA and the oligonucleotides present on the beads. A fluorescent DNA probe that is complementary to the oligonucleotide coupled to the beads is also added under competitive hybridization conditions. Aliquots of the beads so reacted are then run through a flow cytometer and the intensity of fluorescence on each bead is measured to detect the level of fluorescence which indicates the presence or absence of given sequences in the samples.

For example, when beads labeled with an oligonucleotide probe corresponding to a non-mutated (wild-type) DNA segment are hybridized with the PCR product from an individual who has a non-mutated wild-type DNA sequence in the genetic region of interest, the PCR product will effect a significant competitive displacement of fluorescent oligonucleotide probe from the beads and, therefore, cause a measurable decrease in fluorescence of the beads, e.g., as compared to a

control reaction that did not receive PCR reaction product. If, on the other hand, a PCR product from an individual having a mutation in the region of interest is incubated with the beads bearing the wild-type probe, a significantly lesser degree of displacement and resulting decrease in intensity of fluorescence on the beads will be observed because the mutated PCR product will be a less effective competitor for binding to the oligonucleotide coupled to the bead than the perfectly complementary fluorescent wild-type probe. Alternatively, the beads may be coupled to an oligonucleotide corresponding to a mutation known to be associated with a particular disease and similar principles applied. In the multiplexed analysis of nucleic acid sequences, bead subsets are prepared with all known, or possible, variants of the sequence of interest and then mixed to form a bead set. The reactivity of the test sample, e.g. PCR product, with the wild-type sequence and other variants can then be assayed simultaneously. The relative reactivity of the PCR product with subsets bearing the wild-type or variant sequences identifies the sequence of the PCR product. The matrix of information derived from this type of competitive hybridization in which the test sequence and the entire panel of probe sequences react simultaneously allows identification of the PCR product as wild-type, known mutant, or unknown mutant. The invention thus provides one with the ability to measure any of a number of genetic variations including point mutations, insertions, deletions, inversions, and alleles in a simple, exquisitely sensitive, and efficient format.

Figure 1 is a block diagram of an illustrative hardware system for performing a multiplex assay method in accordance with the invention.

Figure 2 is a block diagram of an illustrative software system for performing a multiplex assay method in accordance with the invention.

Figure 3 is a flow-chart for a preprocessing phase in accordance with the inventive multiplexed assay method.

Figure 4 shows an assay database in accordance with the invention.

Figure 5 shows a baseline data acquisition table for an illustrative multiple analyte assay in accordance with the invention.

Figure 6 shows an assay definition table in accordance with the invention.

Figure 7 shows a discriminant table for an illustrative multiple analyte assay in accordance with the invention.

Figure 8 shows a decision tree view of the illustrative discriminant function table of Figure 7.

Figure 9 is a flow-chart for a real-time analysis phase of a multiple analyte assay in accordance with the invention.

Figure 10 shows a results table for an illustrative multiple analyte assay in accordance with the invention.

Figure 11 shows a interpretation table for an illustrative multiple analyte assay in accordance with the invention.

Figure 12 is a flow-chart for an interpretation phase of a multiple analyte assay in accordance with the invention

Figures 13a through 13e show an assay database in accordance with the invention for a specific experimental example.

Figure 14 shows a decision tree view for an illustrative (experimental example) discriminant table.

Figures 15a, 15b, and 15c show individual inhibition assays for IgG, IgA, and IgM antibodies.

Figures 16a, 16b, and 16c show cross reactivity determinations between IgG, IgA, and IgM assay components.

Figure 17 shows the determination of human IgG concentrations by flow cytometry.

Figure 18 shows the determination of human IgA concentrations by flow cytometry.

Figure 19 shows the determination of human IgM concentrations by flow cytometry.

Figure 20 shows the simultaneous determination of human IgG, IgA, and IgM concentrations by flow cytometry.

Figure 21 shows the specificity of monoclonal antibody MAB384 binding towards bead immobilized epitope sequences.

Figure 22 shows the specificity of monoclonal antibody MAB384 binding in the presence of soluble epitope containing peptide.

Figure 23 shows the specificity of monoclonal antibody MAB384 binding in the presence of soluble biotin.

Figure 24 shows the detection of anti-Rubella IgG antibodies by a sandwich assay between rubella coated beads and a fluorescent goat anti-human IgG antibody.

Figure 25 shows a calibration assay using serial dilutions of anti-Rubella IgG antibodies in a sandwich assay between rubella coated beads and a fluorescent goat anti-human IgG antibody.

Figures 26a and 26b show the simultaneous assay for six anti-ToRCH IgG, and simultaneous assay for the six anti-ToRCH IgM antibodies.

Figure 27 shows the determination of IgG anti-grass allergen activities for six dogs.

Figure 28 shows the determination of IgE anti-grass allergen activities for six dogs.

Figure 29 shows the multiple analyte IgG and IgE screening of dog serum A96324 for sixteen grass allergens

Figure 30 shows the multiple analyte IgG and IgE screening of dog serum A96325 for sixteen grass allergens

Figure 31 shows the multiple analyte IgG and IgE screening of dog serum A96319 for sixteen grass allergens

Figure 32 shows the multiple analyte IgG and IgE screening of dog serum A96317 for sixteen grass allergens

Figure 33 shows the multiple analyte IgG and IgE screening of dog serum A96326 for sixteen grass allergens

Figure 34 shows the multiple analyte IgG and IgE screening of dog serum A96323 for sixteen grass allergens

Figure 35 shows an antibody pair analysis for use with a human chorionic gonadotropin capture assay.

Figure 36 shows the use of bead linked antibody MAB602 with fluorescently labeled antibody AB633 in a human chorionic gonadotropin capture assay.

Figure 37a and 37b show cross reactivity analyses between components of an anti-hCG capture system and an anti-AFP capture system.

Figures 38a and 38b compare the effects of eliminating wash steps in hCG and AFP capture system assays.

Figures 39a and 39b show the determination of hCG and AFP concentrations in samples and standards using a homogeneous capture assay format.

Figure 40 shows the inhibition of Anti-IgG binding to bead based IgG by soluble IgG antibodies. Inhibition was determined at five concentrations of soluble IgG, and four IgG loading levels on the beads.

Figure 41 shows the slope of the inhibition pattern across the four loading levels of IgG on the beads plotted against the soluble IgG concentration.

Figure 42 shows a five point standard curve derived from inhibition data of the 50 µg/mL IgG bead set.

Figures 43a through 43c show DNA detection using a double stranded competitor and a wild-type "B" oligonucleotide probe.

Figures 44a and 44b show DNA detection using a single stranded competitor and a wild-type "B" oligonucleotide probe.

Figure 45 shows the differentiation by orange and red fluorescence of fourteen bead sets.

Figure 46 shows a titration of a fluorescent oligonucleotide in the presence or absence of an inhibitor. Beads bearing complementary oligonucleotides were used in a capture assay.

Figure 47 shows the inhibition of binding between a fluorescent oligonucleotide and its complementary oligonucleotide bound to a bead. Varying concentrations of complementary and point mutant competitors were used in the determination.

Figure 48 shows the efficacy of inhibitors across fourteen DNA sequence binding sets.

Figure 49 shows the typing of four simulated alleles of DQA1.

Figure 50 shows the typing of five known, homozygous DQA1 alleles.

Figures 51a through 51f show the results of an exemplary multiplexed assay according to the invention.

According to the present invention, assay components and methods for the measurement of enzymes, DNA fragments, antibodies, and other biomolecules are provided. The inventive technology improves the speed and sensitivity of flow cytometric analysis while reducing the cost of performing diagnostic and genetic assays. Further, and of tremendous significance, a multiplexed assay in accordance with the invention enables the simultaneous automated assay of multiple (at least an order of magnitude greater than available in the prior techniques) biomolecules or DNA sequences in real-time.

As those of ordinary skill in the art will recognize, the invention has an enormous number of applications in diagnostic assay techniques. Beadsets may be prepared, for example, so as to detect or screen for any of a number of sample characteristics, pathological conditions, or reactants in fluids. Beadsets may be designed, for example, to detect antigens or antibodies associated with any of a number of infectious agents including (without limitation, bacteria, viruses, fungi, mycoplasma, rickettsia, chlamydia, and protozoa), to assay for autoantibodies associated with autoimmune disease, to assay for agents of sexually transmitted disease, or to

assay for analytes associated with pulmonary disorders, gastrointestinal disorders, cardiovascular disorders, and the like. Similarly, the beadset may be designed to detect any of a number of substances of abuse, environmental substances, or substances of veterinary importance. An advantage of the invention is that it allows one to assemble a panel of tests that may be run on an individual suspected of having a syndrome to simultaneously detect a causative agent for the syndrome.

Suitable panels may include, for example, a tumor marker panel including antigens such as prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), and other suitable tumor markers; a regional allergy panel including pollen and allergens tested for by allergists of a particular region and comprising allergens known to occur in that region; a pregnancy panel comprising tests for human chorionic gonadotropin, hepatitis B surface antigen, rubella virus, alpha fetoprotein, 3′ estradiol, and other substances of interest in a pregnant individual; a hormone panel comprising tests for T4, TSH, and other hormones of interests; an autoimmune disease panel comprising tests for rheumatoid factors and antinuclear antibodies and other markers associated with autoimmune disease; a blood borne virus panel and a therapeutic drug panel comprising tests for Cyclosporin, Digoxin, and other therapeutic drugs of interest.

Bead Technology

An important feature of the flow cytometric technology and techniques described here is the fabrication and use of particles (e.g., microspheres or beads that make up a beadset). It is through the use of appropriately labeled homogeneous bead subsets, combined to produce a pooled beadset, that the instant multiplexed assay method is practiced. Beads suitable for use as a starting material in accordance with the invention are generally known in the art and may be obtained from manufacturers such as Spherotech (Libertyville, IL) and Molecular Probes (Eugene, OR). Once a homogeneous subset of beads is obtained, the beads are labeled with an appropriate reactant such as a biomolecule, DNA sequence, and/or other reactant. Known methods to incorporate such labels include polymerization, dissolving, and attachment.

A Method for the Multiplexed Assay of Clinical Samples

Development of a multiplexed assay for use in accordance with the invention can be divided into three phases: (1) preprocessing, (2) real-time analysis, and (3) interpretation. During the preprocessing phase, baseline data is collected independently, via flow cytometric techniques, for each of an assay's bead subsets. Baseline data is used to generate a set of functions that can classify any individual bead as belonging to one of the assay's subsets or to a rejection class. During the analysis phase, flow cytometric measurements are used to classify, in real-time, each bead within an exposed beadset according to the aforementioned functions. Additionally, measurements relating to each subset's analyte are accumulated. During the interpretation phase the assay's real-time numerical results are associated with textual explanations and these textual explanations are displayed to a user.

The inventive method allows the detection of a plurality of analytes simultaneously during a single flow cytometric processing step. Benefits of the inventive multiplex assay method include increased speed and reduced cost to analyze a clinical sample.

System Hardware

Figure 1 shows, in block diagram form, a system for implementing the inventive multiplexed assay method. Flow cytometer **100** output consists of a series of electrical signals indicative of one or more specified measured characteristics on each bead processed. These measurement signals are transmitted to computer **105** via data bus **110** and interface board **115**. During the preprocessing phase, the signals are used by the computer to generate an assay database. During the real-time analysis phase, the signals are processed by the computer (using the assay database) in accordance with the inventive method to produce a multiplexed/simultaneous assay of a clinical sample.

Flow cytometer **100** operates in a conventional manner. That is, beads are processed by illuminating them, essentially one at a time, with a laser beam. Measurements of the scattered laser light are obtained for each illuminated bead by a plurality of optical detectors. In addition, if a bead contains at least one appropriate fluorescing compound it will fluoresce when illuminated. A plurality of optical detectors within the flow cytometer measure fluorescence at a

plurality of wavelengths. Typical measured bead characteristics include, but are not limited to, forward light scatter, side light scatter, red fluorescence, green fluorescence, and orange fluorescence. One of ordinary skill in the use of flow cytometric techniques will recognize that the use of green fluorescent markers or labels can cause cross-channel interference between optical detectors designed to detect green and orange wavelengths (e.g., approximately 530 nanometers and approximately 585 nanometers respectively). A training set of beads, in combination with standard data manipulation, can correct for this cross-channel interference by providing the physical measurements required for mathematical correction of the fluorescence measurements.

One of ordinary skill will further recognize that many alternative flow cytometer setups are possible. For instance, additional color sensitive detectors could be used to measure the presence of other fluorescence wavelengths. Further, two or more laser beams can be used in combination to illuminate beads as they flow through the cytometer to allow excitation of fluorochromes at different wavelengths.

Computer **105** can be a conventional computer such as a personal computer or engineering workstation. In one embodiment, the computer is a personal computer having an Intel "486" processor, running Microsoft Corporation's "WINDOWS" operating system, and a number of ISA expansion slots.

Interface board **115** is designed to plug into one of the computer's **100** ISA (Industry Standard Architecture) expansion slots. While the design of an interface board is, in general, different for each specific type of flow cytometer **100**, its primary functions include (1) receiving and parsing measurement data signals generated by the flow cytometer's detectors, (2) receiving control parameter status information from the flow cytometer, and (3) sending control parameter commands to the flow cytometer. The precise manner in which these functions are carried out are dependent upon the type (make and model) of the flow cytometer used. In one embodiment, employing a Becton-Dickinson "FACSCAN" flow cytometer (San Jose, CA), the interface board uses control signals generated by the flow cytometer to distinguish measurement

data and flow cytometer parameter and control signals. Measured data include forward light scatter, side light scatter, red fluorescence, green fluorescence, and orange fluorescence. Parameter and control signals include flow cytometer amplifier gain adjustments and status information.

While the design of an interface board **115** for use with the inventive assay method would be a routine task for one skilled in the art of diagnostic medical equipment design having the benefit of this disclosure, an important aspect for any interface board is its ability to accommodate the transmission data rate generated by whatever flow cytometer is used. For example, the "FACSCAN" flow cytometer can transmit a 16-bit (2 byte) word every 4 microseconds resulting in burst data rates of 500,000 bytes per second. Microfiche appendix A provides a detailed source code embodiment of the inventive assay method for use with the "FACSCAN" flow cytometer.

Data bus **115** provides a physical communication link between the flow cytometer **100** and the interface board **110**. Its physical and electrical characteristics (e.g., data width and bandwidth) are dependent upon the capabilities of the flow cytometer. It is noted that the data bus need not be a totally digital bus. If the flow cytometer does not include analog-to-digital conversion of measured bead characteristics (e.g., light scatter and fluorescence signals), then the data bus must communicate these analog signals to the interface board. It is then necessary that digital conversion of these signals be provided by either the interface board or another peripheral device before the data is transmitted to the computer **105**.

System Software

As shown in **Figure 2**, the software architecture for the inventive assay method can be divided into two parts. A graphical user interface (GUI) **200** provides the means by which a user (1) receives assay results and (2) interacts with the flow cytometer. A dynamically linked library (DLL) **205** provides the means through which the inventive real-time assay is performed and includes routines necessary to (1) interact with interface board **115** and (2) send and receive information to the flow cytometer **100**.

An important aspect of the inventive assay method is that it performs a simultaneous analysis for multiple analytes in real-time. One of ordinary skill in the art of computer software development will realize that real-time processing can impose severe time constraints on the operational program code, i.e., the DLL **205**. For example, the "FACSCAN" flow cytometer can process, or measure, approximately 2,000 beads per second, where each bead is associated with eight 16-bit data values. Thus, to process flow cytometer data in real-time from a "FACSCAN," the DLL should be able to accept, and process, at a consistent data rate of at least 32,000 bytes per second. The need to accommodate this data rate, while also having sufficient time to perform real-time analysis based on the data, will generally necessitate that some of the DLL code be written in assembly language.

In a current embodiment, the GUI **200** is implemented in the visual basic programming language and the DLL **205** is implemented in C and assembly language programming. Microfiche appendix A contains source code listings for one embodiment of the GUI and DLL.

Preprocessing

A function of the preprocessing phase is to generate an assay database for use during the real-time analysis of an exposed beadset (clinical sample). Thus, preprocessing is performed prior to combining separately labeled bead subsets to form assay beadsets. Assay definition, discriminant function definition, and interpretation tables are created at the time an assay beadset is created. **Figure 3** shows, in flow chart form, the steps taken during the preprocessing phase.

A bead subset is characterized by (1) the analyte it is designed to identify, (2) one or more classification parameters $C_1 \ldots C_n$, and (3) one or more measurement parameters $F_{m1} - F_{mx}$. During the preprocessing phase the classification parameters are used to generate a set of functions, referred to as discriminant functions, that can classify a bead as belonging to one of the assay's subsets or a rejection class. Measurement parameters are used during the real-time

analysis phase to determine if a specified analyte is present in the clinical sample being analyzed.

The precise number of individual beads contained in any given subset is relatively unimportant, the only significant criterion being that a sufficient number are used so that a good statistical characterization of the subset's parameters can be achieved during the real-time analysis phase. In a current embodiment, each bead subset contains an equal number of beads. One of ordinary skill in the field will recognize that the precise number of beads within any given bead subset can vary depending upon many factors including, but not limited to, the number of analytes an assay beadset is designed to detect, the uniformity of the labeled beads (with respect to each of the measured parameters $C_1 \ldots C_n$, $F_{m1} \ldots F_{mx}$), and the penalty of misclassifying (e.g., making a type 1 or type 2 classification error) a bead during analysis.

During preprocessing, each bead in an unexposed subset is measured by a flow cytometer **100** and the resulting data values accumulated for later use **300**. For example, if the flow cytometer measures n classification parameters and x measurement parameters, i.e., generates $(n + x)$ values for each bead, data for each of the subset's $(n + x)$ parameters are updated based on each bead's measurements. This data collection step is repeated independently for each subset in the assay's beadset **305**. The collection of such data for each of an assay's subsets constitutes an assay's baseline data.

After an assay's baseline data has been collected, a set of discriminant functions are determined **310**. During real-time analysis, the discriminant functions are used to classify a bead into one of the assay's bead subsets or a rejection class based solely on the measured classification parameters, $C_1 \ldots C_n$. This step, in principle and practice, is a problem of multi-dimensional classification or cluster analysis. Many prior art techniques and commercial software programs exist to perform this task.

Beads are generally manufactured in large quantities referred to as batches. Each bead in a batch is of nearly identical size and has substantially the same dye absorption capacity. In

light of this manufacturing process, bead subsets can be created using precise dilutions of chosen dyes and, because of their nearly identical size, all classification parameters will exhibit essentially equal variances. By correcting for scaling of the photo-multipliers within a flow cytometer, a linear classification rule can be generated. Further, since there are equal quantities of beads in each subset, the prior probabilities will be equal. This allows use of Fisher's linear discriminant technique to calculate the discriminant functions which define classification boundaries. See, Fisher, "The Use of Multiple Measurements in Taxonomic Problems," Annals of Eugenics, 7, 179-188 (1936). For instance, linear hierarchical discriminant functions may be chosen which are equidistant, in a Euclidean sense, between the centers or centroids of any two of an assay's bead subsets. Notwithstanding the present example, other types of discriminant functions, such as quadratic functions and those discriminating on more than two classification parameters at once, are also possible.

In addition to the discriminant functions, a set of threshold values are chosen which are used during the real-time analysis phase to detect the presence of a target analyte. For example, assume measurement parameter F_{mI} is used to detect analyte-A. During preprocessing, the baseline or unexposed value for F_{mI} is measured and accumulated for that subset's beads. Analyte-A's threshold could then, for example, be set to F_{mI}'s baseline mean value plus one standard deviation of F_{mI}'s baseline value. One of ordinary skill will recognize that the precise function or value selected for a threshold depends upon the parameter being measured (e.g., its distribution) and the cost of making a classification error (e.g., a type 1 or a type 2 error). It is routine that such values be based on an empirical review of the baseline data. The important criterion is that the threshold reliably distinguish between the presence and absence of the target analyte in an exposed assay beadset.

After baseline data for each of an assay's bead subsets are collected and discriminant functions and analyte threshold values are established, an assay database is generated **315**.

Assay Database

As shown in **Figure 4**, an assay database **400** consists of an assay definition table **405**, a discriminant function table **410**, a results table **415**, and an interpretation table **420**. See **Figure 4**.

The assay definition table **405** defines an assay which, as described above, comprises two or more bead subsets each of which is designed to detect a specified analyte. Each row in the assay definition table describes a bead subset and contains the following entries: (1) assay name, (2) subset name, (3) subset token, (4) baseline values for each of the subset's measurement parameters $F_{m1} - F_{mx}$, and (5) test-type token. The subset name entry is a text string identifying the subset by, for example, the type of analyte it is labeled to detect. The subset token is a unique subset identifier. The measurement parameter baseline entries are used during the interpretation phase to associate a numerical result (collected during the real-time analysis of a clinical sample) with a textual output string. Finally, the test-type token identifies which one of a possible plurality of interpretation tests to perform on the collected (real-time) data during the interpretation phase.

The discriminant function table **410** is used to systematically set forth an assay's set of discriminant functions. Each row in the discriminant function table implements a single discriminant function and includes entries for (1) the assay's name, (2) a unique row identifier, (3) one or more classification parameters upon which to evaluate, (4) high and low discriminant values for each of the listed classification parameters, and (5) evaluation tokens which are assigned as a result of evaluating the discriminant function.

The results table **415** is used to store, or accumulate, data on an assay's beadset during the real-time analysis phase of the inventive method and is discussed further in Section 6.2(d).

The interpretation table **420** provides a means to associate text messages with each enumerated assay result and is discussed further in Section 6.2(e).

Preprocessing Example

Consider an assay beadset designed to simultaneously detect four analytes: analyte-A, analyte-B, analyte-C, and analyte-D. Thus, the assay's beadset is comprised of four bead subsets, each labeled for a different analyte. Suppose further that the assay beadset is to be processed by a Becton-Dickinson Immunocytometry Systems "FACSCAN" flow cytometer. For each bead processed, the "FACSCAN" measures forward light scatter, side light scatter, red fluorescence, orange fluorescence, and green fluorescence. Let classification parameter C_1 be forward light scatter, classification parameter C_2 be side light scatter, classification parameter C_3 be red fluorescence, classification parameter C_4 be orange fluorescence, and measurement parameter F_{m1} be green fluorescence. (This notation implies that each bead in a subset is labeled with a green fluorophore bearing, for example, an antibody or dye specifically targeted to that subset's analyte.)

After preparing each of the four subsets and before they are combined to form the assay beadset, they are processed by the flow cytometer and their measured data are accumulated: values for each of the parameters C_1, C_2, C_3, C_4, and F_{m1} are recorded for each bead. Each bead subset is similarly processed. Completion of this task constitutes completion of baseline data acquisition.

Using baseline data, the assay's beads are clustered in the four-dimensional parameter space defined by C_1, C_2, C_3, and C_4. The result of this cluster analysis is that each subset is characterized by a mean (μ) and standard deviation (σ) for each of its four classification parameters. See **Figure 5**. As previously noted, the precise number of individual beads contained in any given bead subset can be calculated by those of ordinary skill in the art. This calculation is required to obtain good statistical characterization of the subset's parameters – e.g., small, or relatively fixed, coefficient of variations for each parameter.

As shown in **Figure 6**, the assay definition table **405** is comprised of general information relevant to the overall diagnostic function of the assay. For instance, in a genotyping assay, each of the assay's subset's may be assigned a token used for identification: e.g., token 46 represents

the bead subset labeled to detect a wildtype coding sequence for a specified gene; subset tokens 21, 50, and 5 represent subsets labeled to detect various mutant type coding sequences for a specified gene(s). Additionally, measurement parameter F_{m1}'s baseline (in this example the mean) and standard deviation values are listed. Finally, a test-type token is listed. In the current embodiment a test-type token of '0' means an OVER/UNDER interpretation test is to be performed and a test-type token of '1' means a SHIFT interpretation test is to be performed. See Section 6.2(f) for further discussion of these issues.

Discriminate functions are generated by viewing the assay's baseline data graphically in three dimensions and creating planes to separate the different subset clusters. These "planes" are created by applying Fischer's Linear Discriminant to the *n*-dimensional classification parameter space. A populated discriminate function table based on the baseline data of **Figure 5** is shown in **Figure 7**.

The discriminant function table provides a systematic means of evaluating a series of classification values (C_1, C_2, C_3, C_4) in order to classify a bead. In general bead classification proceeds by entering the discriminant function table at row 0, performing a test on a specified parameter (e.g., C_1, C_2, C_3, or C_4) and then, depending upon the result, either classifying the bead or proceeding to another test which involves evaluating a different row in the table. For example, suppose bead A has the following measured classification parameter values: $C_1 = V_1$, $C_2 = V_2$, $C_3 = V_3$, and $C_4 = V_4$. Classification of bead A via the discriminant function table of **Figure 7** begins as follows (the pseudo-code below would demonstrate to those skilled in the art of programming the logic involved in the classification process):

1. Enter table at row 0 with measured values for C_1, C_2, C_3, and C_4.
2. If (LOW VALUE = 500) ≤ (PARAMETER = C_1 = V_1) ≤ (HIGH VALUE = 620) then (result = TRUE), else (result = FALSE).
3. If (result = TRUE) and (TRUE ROW ID ≠ 0), then re-enter table at TRUE ROW ID, else
4. If (result = TRUE) and (TRUE ROW ID = 0), then (class = TRUE TOKEN).

5. If (result = FALSE) and (FALSE ROW ID ≠ 0), then re-enter table at (row = FALSE ROW ID), else

6. If (result = FALSE) and (FALSE ROW ID = 0), then (class = FALSE TOKEN).

7. If (TRUE TOKEN or FALSE TOKEN) = 0, then (class = reject class).

One of ordinary skill will recognize from the above discussion that a discriminant function table embodies a (classification) decision tree. **Figure 8** shows this relationship for the discriminant function table of **Figure 7** explicitly. A discussion of the discriminant function table as it relates to the real-time processing of an exposed assay beadset is provided in Section 6.2(d). Once a beadset is preprocessed, the data may be employed in real-time analysis of many assays using that set. One of ordinary skill in the art will also recognize that instead of a decision tree, a bitmap or look up table could be used to classify the bead sets.

Real-Time Analysis

Once a collection of bead subsets have been characterized as described above and combined to form an assay beadset, the beadset may be exposed to a test sample. That is, they may be used to analyze a clinical sample. After exposure the beadset is ready for real-time analysis. The real-time analysis phase is initiated by installing the exposed beads into a conventional flow cytometer for processing.

As described above, for each bead processed a flow cytometer **100** generates electrical signals indicative of a plurality of measured parameters, $C_1 \ldots C_n$, $F_{m1} \ldots F_{mx}$. These values are transmitted to computer **105** via data bus **110** and interface board **115**. Values for a bead's classification parameters $C_1 \ldots C_n$ are used to evaluate the assay's discriminant functions, as encoded in a discriminant function table **410**, the result of which is an initial classification of the bead into one of the assay's bead subsets or a reject class.

After this initial classification, a bead's measured classification parameter values $C_1 \ldots C_n$ can be checked against their ($C_1 \ldots C_n$) baseline values to determine if it is "reasonable" to classify the bead as belonging to the initially identified class. In a current embodiment, this

reasonableness test is implemented by computing the distance between the measured classification parameter values and the mean values obtained during preprocessing. If the measured values for $C_1 \ldots C_n$ for a particular bead are sufficiently distant from the identified subsets baseline values, the bead is assigned to a reject class. Use of this technique allows for the rejection of beads that were initially misclassified and improves the overall reliability of the analysis.

To ensure proper classification, a preferred embodiment's pooled beadset will include a bead subset which has no bound reactants (e.g., a placebo bead subset) in a known ratio to the beadset's other subsets.

It is noted that when a beadset is comprised of beads manufactured in a single batch, the above described reasonableness test can be incorporated into the linear discriminant functions by creating reject space between all subsets. However, when a beadset is comprised of beads from more than one batch a Euclidean (or similar) distance measure is needed to validate the classification result.

Once a bead is assigned its final classification, the assay's results table **415** is updated to reflect the newly classified bead's measurement parameter values $F_{m1} \ldots F_{mx}$. This data acquisition, classification, and update process is repeated for each bead in the assay beadset in real-time. **Figure 9** shows, in block diagram form, the general steps performed during the real-time analysis phase of a method in accordance with the invention.

In one embodiment the following data are accumulated in the results table for each class (subset) of bead in the assay: (1) total count of the number of beads detected in the specified class, (2) a running sum for each measurement parameter $F_{m1} - F_{mx}$, (3) for each measurement parameter the total count of the number of beads in the class whose measurement value is less than the parameter's baseline value, and (4) for each measurement parameter the total count of the number of beads in the class whose measurement value is more than the parameter's baseline value.

Real-Time Analysis Example

In the illustrative embodiment introduced in Section 6.2(c), the assay beadset is designed to simultaneously detect four analytes using four classification parameters (C_1 represents forward light scatter, C_2 represents side light scatter, C_3 represents red fluorescence, and C_4 represents orange fluorescence) and one measurement parameter (F_{m1} representing green fluorescence). After exposing the beadset to a suitable biological sample, it is placed into a flow cytometer **100** which processes each bead (e.g., measures parameters C_1, C_2, C_3, C_4, and F_{m1}) and transmits to computer **105** signals indicative of these measurements via data bus **110** and interface board **115**.

For each bead processed by the flow cytometer, values for C_1, C_2, C_3, and C_4 are evaluated in accordance with the discriminant function table shown in **Figure 7** to initially classify the bead as belonging to a particular subset, for example, in a genetic analysis intended to detect mutations in the Kras oncogene, the classification could proceed as follows: (1) class 46, Kras CODON 46 WILDTYPE, (2) class 21, Kras CODON 21 MUTANT, (3) class 50, Kras CODON 50 MUTANT, (4) class 5, Kras CODON 5 MUTANT, or (5) a reject class. (See **Figure 8** for a decision tree representation of the discriminate function table of **Figure 7**.) If the bead is initially classified as belonging to any class except the reject class, a reasonableness test is performed on the bead's classification parameter values, C_1 - C_n. For example, if the bead received an initial classification of class 50 and its C_1 value is more than two standard deviations away from its mean, the bead is given a final classification of reject. Otherwise the bead's final classification is the same as its initial classification – 50.

If the bead's final classification is other than reject, its F_{m1} value is used to update the assay's results table in the following manner (see **Figure 10**):

1. Identifying, based on the bead's classification token (i.e., subset token 46, 21, 50, or 5), the row in the results table which is to be updated.
2. Incrementing the identified row's COUNT value. The COUNT value reflects the total number of beads of the specified class that have been identified during the analysis.

3. Adding the bead's F_{ml} value to the value contained in the row's SUM column. The SUM value reflects a running sum of the identified classes measurement values.
4. If the bead's F_{ml} value is greater than F_{ml}'s base value (determined during the preprocessing phase, see **Figure 6**), then incrementing the row's OVER COUNT value. The OVER COUNT value reflects the total number of beads of the specified class that have been processed whose F_{ml} values are above that of baseline.
5. If the bead's F_{ml} value is less than F_{ml}'s base value (as determined during the preprocessing phase, see **Figure 6**), then incrementing the row's UNDER COUNT value. The UNDER COUNT value reflects the total number of beads of the specified class that have been processed whose F_{ml} values are below that of baseline.

In a preferred embodiment, data (i.e., count, and measured F_{ml} values) for each bead classified as a reject can also be collected.

Interpretation

Following the real-time classification and accumulation of results as described above, the user may select to see a text based presentation or interpretation of the assay's numerical results. During the interpretation phase the assay's real-time numerical results are associated with textual explanations. These textual explanations can be displayed to the user.

It is the function of the interpretation table **420** to associate textual descriptions of an assay's possible outcomes with an actual assay's numerical results. Each row in the interpretation table provides the necessary information to make a single interpretation and typically includes entries for (1) the assay's name, (2) a subset token identifying the class or subset on which the interpretation is based, (3) an outcome identifier for the identified subset, (4) a test-type token, (5) high and low discriminant values for each measurement parameter utilized in the identified test, and (6) a text string describing the row's result.

The test-type token identifies which one of a possible plurality of interpretation tests to perform on the collected (real-time) data during the interpretation phase. In a current

embodiment the test-type token is either '0' or '1'. A value of '0' indicates an OVER/UNDER interpretation test is to be performed. A value of '1' indicates a SHIFT interpretation test is to be performed. These tests are defined in the following manner:

$$\text{OVER/UNDER Test Value} = \frac{OVER\ COUNT}{UNDER\ COUNT}, \text{ and}$$

$$\text{SHIFT Test Value} = \frac{SUM/COUNT}{Baseline\ F_m\ Value},$$

where the variables OVER COUNT, UNDER COUNT, SUM, COUNT, and baseline F_m are described above in Section 6.2(d).

The OVER/UNDER test is generally used for qualitative measurements where the level of reactivity of beads is an indication of the condition or concentration of a biomolecule present in the sample. The shift test is used where the result sought is a determination of the a minimally detectable level of a particular biomolecule. One of ordinary skill will recognize that many other tests could be performed. Examples include ranking, stratification, ratio of means to a standard, or to each other, etc.

In general an interpretation table **420** may associate any number of entries or interpretations (e.g., rows within the table) with a single assay class or bead subset. For instance, bead subset Y could have a single measurement parameter (F_{m1}) associated with it and this measurement parameter could indicate, depending upon its value, that one or more interpretations are appropriate.

Note, the contents of the interpretation table **420** are generated during the preprocessing phase. This implies that the target assay be understood and that the various assay results be considered prior to construction of multiplexed assays.

Interpretation Example

Consider again the assay beadset, introduced above, designed to simultaneously detect four analytes. **Figure 11** shows a sample interpretation table for this assay. Interpretation of the assay's real-time numerical results is initiated by, for example, the user selecting "interpret results" via the inventive method's graphical user interface.

As described above, each bead subset (class) within an assay has an entry or row in the results table, **Figure 10**. The general procedure for interpreting an assay's real-time numerical results is shown in flow-chart form in **Figure 12**. In general, each row of the results table is matched against every row in the interpretation table with the same subset token. If the result of performing the specified test is between the identified row's low and high values, then the associated textual message is displayed to the user. When all rows in the interpretation table for a single results table row have been checked, the next results table row is evaluated. This process is repeated until the every row in the interpretation table has been compared to the appropriate results table entry.

As a specific example, consider the interpretation of subset 50's (KRAS CODON 50 MUTANT, see **Figure 6**) results table entry. The subset's token, 50, is used to identify three rows in the interpretation table (having outcome IDs of 1, 2, and 3) that contain information regarding evaluation of the mutant analyte. For the first identified row, the test-type token indicates a SHIFT type interpretation test is to be performed. Performing this test, as defined above, yields:

$$\text{SHIFT Test Value} = \frac{SUM/COUNT}{Baseline\ F_m\ Value} = \frac{1{,}700{,}000/1{,}000}{170} = 10$$

Next, the computed SHIFT test value is compared against each interval in the identified rows of the interpretation table. For the row having OUTCOME ID equal to 1, since (LOW VALUE = 10) ≤ SHIFT Test Value = 10 ≤ (HIGH VALUE = 667) is true, that row's INTERPRETATION entry – "identical complementary strand" – is displayed to the user. This

process is repeated for subset 50's remaining two rows in the interpretation table. Further, this process is repeated for each row in the results table.

The result of the interpretation phase is a series of textual messages that describe the results of the assay. Conclusion of the interpretation phase marks the end of the assay.

Operational Considerations

Assay definition, discriminant function definition, and interpretation tables are created at the time an assay beadset is created. Baseline classification data is collected only once for a given assay. That is, once an assay is defined and its baseline data is obtained, any number of beadsets can be manufactured to perform the analysis. To allow this "sharing" of baseline data the assay beadset may contain a center or calibration bead subset.

As would be known to those of ordinary skill in the field, a calibration beadset can be used to adjust any given flow cytometer to a standard. Calibration beadsets are typically processed separately from an assay. Further, calibration is generally performed daily. The purpose of calibration is to adjust the sensitivity of a flow cytometer's photomultipliers to accommodate day to day and machine to machine differences.

Unlike prior art calibration techniques which are performed manually, the processing of a calibration beadset and the adjustment of flow cytometer operational parameters (e.g., photomultiplier voltages) is performed under software control automatically. See microfiche appendix A for embodiment details.

Antibody Detection

Assays for antibody are widely used in medicine and clinical analysis for an wide variety of purposes, from detection of infections to determination of autoantibody. The following example illustrates use of the inventive method in an antibody assay and assumes the use of a flow cytometer capable of providing at least five measurements for each bead processed: forward light scatter as classification parameter C_1, side light scatter as classification parameter

C_2, red fluorescence as classification parameter C_3, orange fluorescence as classification parameter C_4, and green fluorescence as measurement parameter F_{m1}.

In one method a number of bead subsets, e.g., subsets 1 through 10 (identified as sS1-sS10), are prepared, for example, by using a cell sorter to sort a heterogeneous population to collect a homogeneous subset or alternatively, by preparing the beads using tightly controlled specifications to ensure production of a homogeneous subset. Each subset is distinguishable by its characteristic pattern of classification parameters C_1, C_2, C_3, and C_4. The beads in each subset are then labeled with a different antigen such as AgA, AgB, etc. so as to create a collection of labeled subsets as follows: sS1-AgA, sS2-AgB, sS3-AgC, sS4-AgD, sS5-AgE, sS6-AgF, sS7-AgG, sS8-AgH, sS9-AgI, and sS10-AgJ.

Antigens AgA through AgJ may be attached to the beads by any of a number of conventional procedures such as by chemical or physical absorption as described by Colvin et al., "The Covalent Binding of Enzymes and Immunoglobulins to Hydrophilic Microspheres" in Microspheres: Medical and Biological Applications, 1-13, CRC, Boca Raton, FL, 1988; Cantarero et al., "The Adsorptive Characteristics of Proteins for Polystyrene and Their Significance in Solid-Phase Immunoassays," Anal. Biochem., 105, 375-382 (1980); and Illum et al., "Attachment of Monoclonal Antibodies to Microspheres," Methods in Enzymol., 112, 67-84 (1985).

After attachment of antigen to the beads' surface, aliquots from each subset are mixed to create a pooled or assay beadset, containing known amounts of beads within each subset. Preferably, the pooled set is prepared with equal volumes of beads from each subset, so that the set contains about the same number of beads from each subset.

The assay beadset may then be incubated with a fluid sample of interest, such as serum or plasma, to test for the presence of antibodies in the fluid that are reactive with antigens on the beads. Such incubation will generally be performed under conditions of temperature, pH, ionic concentrations, and the like that facilitate specific reaction of antibodies in the fluid sample with

antigen on the bead surface. After a period for binding of antibody, the beads in the mixture are centrifuged, washed and incubated (again under controlled conditions) for another period of time with a "secondary" antibody such as, for example, fluorescein labeled goat anti human immunoglobulin. The secondary antibody will bind to and fluorescently label antibodies bound to antigen on the beads. Again after washing (or without washing), the beads are processed by the flow cytometer and the four classification parameters forward light scatter , side light scatter, red fluorescence, and orange fluorescence are measured and used to identify the subset to which each bead in the assay beadset belongs. A simultaneous measurement of green fluorescence (measurement parameter) for each bead allows one to determine whether the bead has antibody bound to it. Because the subset to which a bead belongs is correlated with the presence of a particular antigen, e.g., sS1-AgA, one may readily determine the specificity of the antibody bound to a bead as a function of the subset to which it belongs.

Experimental Example

Three different antigen-antibody pairs were used in a multiplex experiment demonstrating the ability to detect the presence or absence of several antibodies in a single sample. Antigens were coupled to latex microspheres via carbodiimide coupling, and the corresponding antibodies were fluorescently labeled with fluorescein isothiocyanate (green fluorescence – F_m). Each antigen was coupled to a unique microsphere. Baseline data for the fluorescent antibodies and antigen-microsphere complexes used in this experiment are shown in **Figure 13a**. Baseline data for the three bead subsets of **Figure 13a** are given in **Figure 13b**.

The absence of fluorescence (C_2 and C_3) immediately discriminates the clear beads (subset 50) from beads in the other two subsets. Subsets 45 and 50 were further discriminated by side light scatter (C_1) and red fluorescence (C_3). Linear discriminant functions based on these observations and created as described in Section 6.2(c); are shown in **Figure 13c**. Accepting only clear beads with side light scatter (C_1) within ± 0.25 standard deviations of the mean, doublets (two beads stuck together) were eliminated from the analyses. The remaining beads were classified by red fluorescence (C_3) at a midpoint of 59.6. A decision tree based on the discriminant function table (**Figure 13c**) is shown in **Figure 14**.

In this experiment, each of four samples (e.g., blood serum from four patients) contained all three antigen-microsphere complexes and either 1 or 2 different fluorescent antibodies in PBS buffer. After addition of the antibodies, the reactions were incubated at room temperature for 45 minutes, and then analyzed on the "FACSCAN" using side light scatter (C_1), orange fluorescence (C_2), and red fluorescence (C_3) as classification parameters. Green fluorescence was used as the measurement parameter (F_m); an increase in green fluorescence by 30-fold indicates a specific interaction between an antigen and its corresponding fluorescinated antibody. In other words, if a subset's mean measured F_m value is greater than 30-fold times that subset's baseline F_m value, then the target analyte is determined to be present. These "interpretive" observations are embodied in the interpretation table shown in **Figure 13d**.

Once the assay database was built, it was tested by running 5,000 beads from each bead subset individually through the system. After rejecting 23.8% of the beads as doublets, the remaining crimson beads (subset 18) were classified with 99.88% accuracy. Dark red beads (subset 45) were classified with 99.96% accuracy with 22.9% rejected as doublets. Clear beads (subset 50) were classified with 100% accuracy with 9.4% of the beads rejected as doublets.

The three bead subsets were pooled to form an assay beadset and divided into 4 sample tubes and processed by the system shown in **Figure 1**. The contents of each sample and the mean measured fluorescence (F_m) for each bead subset are listed in **Figure 13e**. The inventive method correctly identified the antibody or antibodies present in each sample.

An Experimental Refinement

In an alternative embodiment using a C_4 (e.g., orange fluorescence) labeled reactant as a classification parameter, a variety (for example five) of protein antigens are employed. Bead subsets are first generated based on differences in one or more of C_1, C_2, and C_3. Next, a selected antigen labeled with Cy3NHS (an orange fluorophore) is bound to the beads in each subset. To minimize the measured orange fluorescence coefficient of variation for each bead subset, the beads are sorted with a high speed cell sorter so that only a narrow range of antigen

(orange fluorophore) is found on each bead within a subset. Care should be taken to select or prepare the beadset so that different C_4 values are measured/obtained for each of the (e.g., five) different antigens used. In other words, the measured intensity of C_4 for AgA should differ from the measured intensity of C_4 from AgB, etc. To ensure that uniformity is achieved, saturation binding with fluoresceinated monoclonal antibody is tested – each bead ought to have restricted ranges of both orange and green fluorescence. While the construction of beadsets by this method is more laborious, the increase in measurement precision may be useful and will allow the sampling of fewer beads to arrive at a suitable determination of antibody concentration.

The assays previously mentioned measure any antibody with specificity for antigen upon an appropriately labeled bead. The antigen can be quite simple or rather complex and thus, the inventive methods can measure a highly restricted antibody or a broad array of antibodies. For example, a hexapeptide just large enough to bind to a monoclonal antibody can be employed as antigen or a large protein with many epitopes can be used. One of ordinary skill will recognize that the level of antibody eventually found associated with the bead (F_{ml}) is a function of the number of epitopes per bead, the concentration of epitopes, the amount of antibody and the affinity of the antibody and the valence of the antibody-antigen interaction.

Displacement Assays

Assays for many substances in a clinical laboratory are based on the interference with specific ligand-ligate or antigen-antibody interactions. In these assays, one member of the ligand-ligate pair is labeled with the F_m fluorophore and one member is immobilized on the beads. Soluble, unlabeled material (analyte) ,which may be ligand or ligate, is added to the reaction mixture to competitively inhibit interaction of the labeled component with the immobilized component. It is usually not important which member of the pair is labeled and which is immobilized; however, in certain assays, functional advantages may dictate the orientation of the assay.

In an exemplary assay of this type, each bead subset is modified with an antigen. The antigen-coated beads are then reacted with an F_m labeled antibody specific for the antigen on the

bead surface. Subsequent addition of a test fluid containing soluble analyte (inhibitor) will displace the F_m labeled antibody from the beads in direct proportion to the concentration of the soluble analyte. A standard curve of known analyte concentrations is used to provide accurate quantification of analyte in the test sample.

One of ordinary skill will recognize that the time necessary to achieve equilibrium may be quite lengthy due to the kinetics and association constant of the interaction. To lessen the time required for the assay, the fluid containing the beadset may be subjected to dissociating conditions such as a change in pH, ionic strength or temperature, after mixture of the beadset with the sample to be tested. Alternatively, the F_m labeled component may be added to the beadset after addition of the test sample. In either case, it is not necessary for equilibrium to be achieved to determine analyte concentration if the kinetics and linearity of the assays have been established.

Additional Experimental Examples

The following series of experimental examples illustrates how the above referenced techniques can be used in practice in effective diagnostic assays. In one embodiment for example, a competitive inhibition analysis is used to quantitate levels of selected analytes, here IgG, IgA, and IgM. A second experimental refinement demonstrates the utility of multiplexed assays in epitope mapping of a monoclonal antibody. In one embodiment, that approach involved the use of antibody detection technology using a fluoresceinated monoclonal antibody in combinatorial epitope screening (e.g. of peptide libraries) to map a particular epitope to which a monoclonal antibody of interest bound, together with a displacement (competitive inhibition) aspect to demonstrate the specificity of the assay. Also described is a ToRCH assay for screening of human serum for antibodies to a number of infectious agents known to pose special hazards to pregnant women. Allergy screening is exemplified by detection of serum IgE against a panel of grass antigens. Yet an additional experimental example reflects the ability of the multiplexed assay in pregnancy testing, e.g. in testing for hormones or other analytes commonly elevated during pregnancy. Each of these examples is set forth below.

Simultaneous competitive inhibition assay of human immunoglobuling G, A and M levels in serum

This example illustrates the determination of multiple analyte levels in a liquid sample simultaneously using competitive inhibition analysis. The use of a competitive inhibition assay to accurately determine analyte levels in liquid solutions is a commonly used format for many analyte assays. The uniqueness of this assay is the simultaneous determination of three distinct serum proteins at the same time in the same tube from one serum sample.

Immunoglobulins G, A and M are three distinct serum proteins whose levels are determined by a number of genetic and environmental factors in human serum. As changes to these levels may indicate the presence of disease, clinicians often request assay determinations of IgG, A and M using conventional techniques. The most common technique is nephelometry that depends upon the absorption of light by precipitates formed between these immunoglobulins and antibodies made in animals to the human immunoglobulins. As these immunoglobulins are present in human serum at fairly high levels, this type of assay is sufficient. Nephelometry however suffers from a number of limitations including the need for large quantities of reagents, long reaction times for precipitation to equilibrate and an inability to perform more than one reaction per tube or sample.

Three competitive inhibition assays are described, one for human IgG, one for human IgM and one for human IgA using three Differentially Fluorescent Microspheres (DFM). Each assay consists of a DFM coated with the immunoglobulin of choice and a polyclonal, goat anti-human Ig labeled with a green fluorescent molecule (Bodipy). In the absence of inhibitor, the Bodipy -antibody causes the immunoglobulin (Ig) coated microsphere to emit green fluorescence (F_m). In the presence of inhibitor (soluble Ig), the green signal is reduced. Each assay is balanced to reflect a sensitivity range near the physiological level of the Ig in question at a 1:500 dilution of human serum. Once balanced, the three assays were combined into a multiple analyte format and assayed simultaneously using flow cytometry.

Antibody labeling: Goat anti-human IgG, goat anti-human IgA, and goat anti-human IgM antibodies (Cappel Division, Organon Teknika, Durham, NC) were labeled with Bodipy FL-CASE (Molecular Probes, Inc., Eugene, OR) using methods described by the manufacturer of the Bodipy succinymidyl ester. The resulting Bodipy labeled antibodies were stored in PBS containing 1 mg/mL BSA as stabilizer.

Antigen conjugation to microspheres: Four DFM (5.5 μM carboxylate, Bangs Laboratories, Inc. (Carmel, IN), dyed by Emerald Diagnostics, Inc. (Eugene, OR)) were conjugated separately to human IgG, human IgA, human IgM (Cappel Division, Organon Teknika, Durham, NC) and BSA with a two-step EDC coupling method (Pierce Chemicals, Rockford, IL) using sulfo-NHS to stabilize the amino-reactive intermediate. 100 μL of each bead type (4.2×10^7 microspheres) was activated for 20 minutes in a total volume of 500 μL containing 500 μg of EDC and Sulfo-NHS in 50 mM sodium phosphate buffer, pH 7.0. The microspheres were washed twice with 500 μL PBS, pH 7.4 using centrifugation at 13,400 x g for 30 seconds to harvest the microspheres. Activated, washed beads were suspended in 250 μL of a 0.05 mg/mL solution of protein in PBS, pH 7.4. After 1 hour, the microspheres were blocked by addition of 250 μL of 1.0 mg/mL BSA, 0.02% Tween, 0.2 M glycine, in PBS, pH 7.4 and incubated for an additional 30 minutes. Protein coated microspheres were washed twice with 500 μL 0.02% Tween 20, 1 mg/mL BSA in PBS, pH 7.4 (PBSTB). and stored in PBSTB at approximately 3,000,000 microspheres/mL. Microsphere concentrations were determined using a hemacytometer.

Determination of appropriate ranges of quantitation for each Ig assay: The normal range of human Ig levels in serum as reported in Clinical Chemistry: Principles and Technics, 2nd Edition, Edited by R.J. Henry, D.C. Cannon and J.W. Winkleman are 569-2210 mg/dL for IgG, 51-425 mg/dL for IgA and 18-279 mg/dL for IgM. Each inhibition assay was designed to be sensitive to inhibition across these ranges.

Single analyte assay: 10 μL of dilutions of a serum calibrator with known Ig levels (Kamiya Biomedical, Thousand Oaks, CA) was first mixed with 10 μL of Ig loaded microspheres containing 7,500 beads. Next, 10 μL of the Bodipy-labeled Goat Anti-Ig was added and the

mixture incubated at ambient temperature for 30 minutes. The mixture was diluted to 300 μL in PBSTB and assayed by flow cytometry. For IgG, the Bodipy-labeled goat anti-hIgG was used at 30 μg/mL. For IgA, the Bodipy-labeled goat anti-hIgA was used at 8 μg/mL. For IgM, the Bodipy-labeled goat anti-hIgM was used at 2.5 μg/mL.

Cross reactivity assay: Equivalent amounts of each of the four protein loaded microspheres were mixed to produce a bead mixture. 10 μL of the bead mixture (7,500 microspheres) was mixed with 10 μL of diluted serum calibrators of known Ig level. The assay was initiated by addition of 10 μL of one of the Bodipy-labeled antibodies "spiked" with a small quantity of soluble Ig antigen to alleviate the "hook effect". The mixtures were incubated for 30 minutes, diluted to 300 μL in PBSTB and assayed by flow cytometry. As before for the single analyte assay, the Bodipy-labeled goat anti-hIgG was used at 30 μg/mL. For IgA, the Bodipy-labeled goat anti-hIgA was used at 8 μg/mL. For IgM, the Bodipy-labeled goat anti-hIgM was used at 2.5 μg/mL. The quantities of antigen "spikes" were 1.6 μg/mL for IgG, 0.6 μg/mL for IgA and 0.4 μg/mL for IgM.

Multiple analyte assay: Equivalent amounts of each of the four protein loaded microspheres were mixed to produce a bead mixture. 10 μL of the bead mixture (7,500 microspheres) was mixed with 10 μL of diluted serum calibrators of known Ig level as well as three other calibrator sera of known Ig level to serve for this purpose as unknowns. The assay was initiated by addition of 10 μL of a mixture of the three Bodipy-labeled antibodies "spiked" with a small quantity of the three soluble Ig antigen to alleviate the "hook effect". The mixtures were incubated for 30 minutes, diluted to 300 μL in PBSTB and assayed by flow cytometry. As before, the Bodipy-labeled goat anti-hIgG was used at 30 μg/mL. For IgA, the Bodipy-labeled goat anti-hIgA was used at 8 μg/mL. For IgM, the Bodipy-labeled goat anti-hIgM was used at 2.5 μg/mL. The quantities of antigen "spikes" were 1.6 μg/mL for IgG, 0.6 μg/mL for IgA and 0.4 μg/mL for IgM.

Results

IgG single analyte assay: Results of the single analyte inhibition analysis for IgG level is shown in **Table 1** and **Figure 15A**. This assay was designed to be most sensitive to inhibition in the anticipated range of IgG in human serum at a 1:500 dilution. In **Figure 15A**, the area of the inhibition curve between the dotted lines, left and right, cover the range of sensitivity. In this case, the inhibitor was known amounts of human IgG from a serum calibrator diluted into human serum containing no IgG, IgA or IgM. Dilutions of the calibrator were then diluted 1:500 in PBSTB and included as inhibitor in the assay. The Bodipy-labeled anti-hIgG was used at 30 μg/mL in PBSTB. 7,500 microspheres were used in this experiment and 250 were counted by flow cytometry. Note that as the amount of soluble IgG increased, the degree of inhibition as monitored by the MIF of F_m increased proportionally until saturation of the system was achieved. On the other end of the inhibition curve note that the lower levels of soluble inhibitor caused an elevation in the MIF of F_m as compared with the negative control (human serum with no Ig). This "hook effect" is common in immunoassay and can be adjusted up or down the inhibition curve by adjusting both the amount of antibody and antigen in the soluble portion of the assay. The "hook effect" was most prominent in the IgG assay due to the higher concentrations of both antigen and antibody per microsphere. This was necessary as IgG is found in serum at higher concentrations than IgA or IgM.

IgA single analyte assay: Results of single analyte inhibition analysis for IgA level is shown in **Table 1** and **Figure 15B**. This assay was designed to be most sensitive to inhibition in the anticipated range of IgA in human serum at a 1:500 dilution. In **Figure 15B**, the area of the inhibition curve between the dotted lines, left and right, cover the range of sensitivity. In this case, the inhibitor was known amounts of human IgA from a serum calibrator diluted into human serum containing no IgG, IgA or IgM. Dilutions of the calibrator were then diluted 1:500 in PBSTB and included as inhibitor in the assay. The Bodipy-labeled anti-hIgA was used at 8 μg/mL in PBSTB. 7,500 microspheres were used in this experiment and 250 were counted by flow cytometry. Note that as the amount of soluble IgA increased, the degree of inhibition as monitored by the MIF of F_m increased proportionally until saturation of the system was achieved. On the other end of the inhibition curve note that the lower levels of soluble inhibitor

cause a slight elevation in the MIF of F_m as compared with the negative control (human serum with no Ig). The "hook effect" was much less pronounced for both IgA and IgM due to their lower concentrations in serum.

IgM single analyte assay: Results of single analyte inhibition analysis for IgM level is shown in **Table 1** and **Figure 15C**. This assay was designed to be most sensitive to inhibition in the anticipated range of IgM in human serum at a 1:500 dilution. In **Figure 15C**, the area of the inhibition curve between the dotted lines, left and right, cover the range of sensitivity. In this case, the inhibitor was known amounts of human IgM from a serum calibrator diluted into human serum containing no IgG, IgA or IgM. Dilutions of the calibrator were then diluted 1:500 in PBSTB to be included as inhibitor in the assay.. The Bodipy-labeled anti-hIgM was used at 2.5 μg/mL in PBSTB. 7,500 microspheres were used in this experiment and 250 were counted by flow cytometry. Note that as the amount of soluble IgM increased, the degree of inhibition as monitored by the MIF of F_m increased proportionally until saturation of the system was achieved. On the other end of the inhibition curve note that the lower levels of soluble inhibitor cause a slight elevation in the MIF of F_m as compared with the negative control (PBS with no added IgM). The "hook effect" is much less pronounced for both IgA and IgM due to their lower concentrations in serum.

Cross reactivity analysis: To determine the cross-reactivity of the various assay components, a multiple analyte assay was performed using only one of the three Bodipy-labeled antibodies. Equivalent numbers of the IgG, IgA, IgM and BSA beads were mixed to make a GAM mixed bead set. To 10 μL of the bead set (7,500 microspheres) was added 10 μL of dilutions of the calibrator containing IgG, IgA and IgM. The multiple analyte assay was then performed using only one of the Bodipy-labeled anti-IgG, IgA or IgM preparations rather than a mixture. **Table 2** and **Figures 16A, 16B,** and **16C** show the results of these assays. Results indicated that Anti-IgG-Bodipy only reacted with DFM-IgG Bodipy and not the IgA or IgM beads. No cross-reactivity with IgA or IgM was noted and the assay was validated for further multiple analyte analysis. Also added to this analysis was the antigen "spike". By adding a small amount of soluble antigen to the probe antibody solution the "hook effect' can be minimized. Note in the

IgG cross-reactivity experiment that the MIF of F_m for negative control is higher than the lowest concentration of inhibitor. By spiking the experiment with 1.6 μg/mL IgG the hook effect has no effect at the lower end of inhibitor range leading to a more accurate assay over the entire dynamic range.

GAM simultaneous analysis: Equivalent numbers of the IgG, IgA, IgM and BSA beads were mixed to make a GAM mixed bead set. To 10 μL of the bead set (7,500 microspheres) was added 10 μL of dilutions of the calibrator containing IgG, IgA and IgM. Also included were several additional calibrators that served as unknowns for the demonstrative purpose of this assay. The multiple analyte assay was then initiated by adding 10 μL of a mixture of the Bodipy-labeled anti-IgG, IgA and IgM plus the soluble Ig "spikes". After a 30 minute, room temperature incubation the reaction mixture was diluted to 300 μL and 1000 microspheres counted by flow cytometry. **Tables 3-5** and **Figures 17-19** show the results of these assays. For each of the inhibition curves produced, a polynomial trendline was used as a non-linear regression analysis. The fit of this trendline to the data was demonstrated by the R^2 correlation factor (1.0 is a perfect fit). The factors of the polynomial formula were used to predict the quantity of inhibitor in each dilution of calibrator and "unknown" serum. The differences between the predicted inhibitor quantities and actual amounts were also included in **Tables 3-5**. Results indicate that this multiple analyte inhibition assay can determine the level of these 3 serum proteins with an error of less than 10 %. Coefficients of variation (CV) between the triplicate data points indicated that the assay was highly precise (no CV greater than 6%). Limits of quantitation for each assay were 400 - 3000 mg/dL for IgG, 60- 455 mg/dL for IgA, and 36 - 272 mg/dL for IgM. **Figure 20** shows the results of the three assays graphically represented on the same graph as all three assays were performed at the same time in the same tube.

A multiple analyte, competitive inhibition assay for human serum IgG, IgA, and IgM levels has been developed. This assay, that allows the simultaneous assay of these three protein levels in serum diluted 1:500, demonstrated excellent sensitivity, precision and accuracy.

TABLE 1: Single analyte inhibition assays

	IgG	MIF of		**IgA**	MIF of		**IgM**	MIF of
Tube#	**mg/dL**	*Fm*		**mg/dL**	*Fm*		**mg/dL**	*Fm*
1	0	1445		0	1654		0	1765
2	0.026	1500		0.0040	1645		0.0024	1794
3	0.11	1460		0.016	1729		0.010	1929
4	0.42	1512		0.064	1734		0.038	1921
5	1.7	1426		0.26	1733		0.15	1815
6	6.8	1619		1.02	1747		0.61	1829
7	27.1	1684		4.1	1746		2.4	1833
8	108	1943		16.4	1788		9.8	1807
9	163	1898		24.6	1813		14.7	1792
10	244	1885		36.9	1806		22.0	1723
11	366	1624		55.3	1703		33.0	1704
12	549	1456		83.0	1391		49.6	1446
13	824	998		125	971		74.3	1267
14	1235	722		187	558		112	879
15	1853	473		280	336		167	591
16	2779	350		420	240		251	360
17	4169	313		630	140		376	269
18	6253	316		945	103		564	242
19	9380	196		1418	75		847	136
20	14070	165		2127	54		1270	102

TABLE 2: Cross-reactivity analysis in multiple analyte assay

1) GAM Beads reacted with **anti-IgG -Bodipy @ 30 µg/mL** + Ag spikes.

Tube	hu IgG mg/dL	**Bead 1- MIF HuIgG**	hu IgA mg/dL	**Bead 2- MIF HuIgA**	hu IgM mg/dL	**Bead 3- MIF HuIgM**	**Bead 4- MIF BSA**
1	0	**1868**	0	4	0	6	5
2	400	**1702**	60.5	4	36.1	7	5
3	561	**1463**	84.7	4	50.6	5	5
4	785	**1218**	119	3	70.8	4	5
5	1099	**880**	166	3	99.2	3	5
6	1538	**674**	233	3	139	3	5
7	2154	**549**	326	2	194	3	5
8	3015	**450**	456	2	272	2	5

2) GAM Beads reacted with **anti-IgA -Bodipy @ 8 µg/mL** + Ag spikes.

11	0	3	0	**1800**	0	2	3
12	400	2	60.5	**1455**	36.1	2	3
13	561	8	84.7	**1225**	50.6	1	3
14	785	3	119	**930**	70.8	1	3
15	1099	2	166	**605**	99.2	1	3
16	1538	2	233	**392**	139	1	3
17	2154	2	326	**278**	194	1	3
18	3015	2	456	**163**	272	1	3

3) GAM Beads reacted with **anti-IgM -Bodipy @ 2.5 μg/mL** + Ag spikes.

21	0	3	0	6	0	**1536**	2
22	400	3	60.5	9	36.1	**1284**	2
23	561	3	84.7	2	50.6	**1135**	2
24	785	3	119	2	70.8	**1011**	2
25	1099	2	166	2	99.2	**776**	2
26	1538	2	233	1	139	**620**	2
27	2154	2	326	2	194	**463**	2
28	3015	2	456	1	272	**330**	2

TABLE 3: Multiple analyte IgG inhibition data

Tube #	hIgG mg/dL	MIF of *Fm*	Average MIF	MIF CV	Calculated mg/dL	% Difference
1		1917				
2	**0**	1943	**1926**	0.6%	**na**	na
3		1918				
4		1811				
5	**400.4**	1737	**1772**	1.7%	**399.3**	0.3%
6		1767				
7		1408				
8	**560.6**	1529	**1471**	3.4%	**566.9**	-1.1%
9		1476				
10		1250				
11	**784.8**	1163	**1236**	4.4%	**775.3**	1.2%
12		1295				
13		852				
14	**1099**	867	**862**	0.8%	**1102.5**	-0.3%
15		868				
16		661				
17	**1538**	726	**691**	3.9%	**1556.1**	-1.2%
18		687				
19		575				
20	**2154**	575	**580**	1.1%	**2126.3**	1.3%
21		589				
22		461				
23	**3015**	466	**468**	1.5%	**3025.1**	-0.3%
24		478				

"UNKNOWNS"

25		1691				
26	**446**	1657	**1657**	1.7%	**411.3**	7.8%
27		1624				
28		749				
29	**1243**	737	**763**	3.8%	**1316.8**	-5.9%
30		804				
31		464				
32	**3045**	486	**476**	1.9%	**2947.1**	3.2%
33		479				

TABLE 4: Multiple analyte IgA inhibition data

Tube #	hIgA mg/dL	MIF of *Fm*	Average MIF	MIF CV	Calculated mg/dL	% Difference
1		1954				
2	**0**	1941	**1952**	0.4%	**na**	na
3		1960				
4		1661				
5	**60.5**	1664	**1665**	0.2%	**60.5**	0.0%
6		1669				
7		1222				
8	**84.7**	1391	**1307**	5.3%	**84.7**	0.0%
9		1308				
10		1055				
11	**118.6**	974	**1051**	5.9%	**118.6**	0.0%
12		1125				
13		615				
14	**166.1**	595	**606**	1.4%	**166.1**	0.0%
15		607				
16		376				
17	**232.5**	426	**400**	5.1%	**232.6**	0.0%
18		399				
19		283				
20	**325.5**	280	**287**	2.9%	**325.4**	0.0%
21		299				
22		193				
23	**455.7**	198	**195**	1.2%	**455.7**	0.0%
24		193				

"UNKNOWNS"

25		1569				
26	**65**	1483	**1504**	3.1%	**68.2**	-4.9%
27		1460				
28		455				
29	**187**	457	**477**	6.1%	**197.2**	-5.5%
30		518				
31		187				
32	**454**	199	**201**	5.9%	**445.4**	1.9%
33		216				

TABLE 5: Multiple analyte IgM inhibition data

Tube #	hIgM mg/dL	MIF of *Fm*	Average MIF	MIF CV	Calculated mg/dL	% Difference
1		1566				
2	**0**	1615	**1605**	1.8%	**na**	na
3		1635				
4		1345				
5	**36.1**	1312	**1328**	1.0%	**35.7**	1.2%
6		1328				
7		1133				
8	**50.6**	1182	**1155**	1.8%	**52.9**	-4.6%
9		1151				
10		1038				
11	**70.8**	994	**1035**	3.2%	**68.1**	3.9%
12		1074				
13		728				
14	**99.2**	733	**735**	0.9%	**100.7**	-1.5%
15		744				
16		514				
17	**138.8**	585	**546**	5.4%	**138.3**	0.4%
18		539				
19		424				
20	**194.4**	414	**419**	1.0%	**194.0**	0.2%
21		418				
22		298				
23	**272.1**	339	**315**	5.6%	**272.4**	-0.1%
24		307				

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25		1266				
26	**40**	1248	**1241**	1.9%	**42.8**	-7.0%
27		1209				
28		608				
29	**113**	621	**635**	4.7%	**116.2**	-2.8%
30		677				
31		289				
32	**268**	315	**306**	3.9%	**281.0**	-4.9%
33		313				

Epitope Mapping of a Monoclonal Antibody using Flow Cytometry.

This example demonstrates the screening of combinatorial chemistry products for a biologically active molecule. The generation of random chemical products for empirical discovery of biologically significant molecules is a method that holds great promise for progress in numerous disciplines of science including biology, pharmacology and medicine. One general problem with the technique is the screening of large numbers of unique molecules for a specific activity. Screening methods are required that provide high throughput levels of screening with adequate specificity and sensitivity for detection of the biological event in question.

An experiment was designed to demonstrate the screening of peptides for the epitope of a monoclonal antibody. A monoclonal antibody (MAB 384) was chosen that was produced using the spleen cells of a mouse hyper-immunized with a defined peptide (amino acid 67-74) from the amino acid sequence of human myelin basic protein (MBP). Using the amino acid sequence of this region of MBP, nine overlapping octapeptides were synthesized that covered the predicted epitope. To the carboxyl terminal end of each peptide, glycine-lysine-biotin residues were added. Nine Differentially Fluorescent Microspheres (DFM) were each coated with avidin and one unique peptide of the set was linked through the avidin-biotin interaction to one unique member of the bead set. This resulted in a set of microspheres that contained nine

members each carrying a unique peptide either flanking or representing the monoclonal antibody's epitope. The bead carrying the epitope peptide was detected using the MAB 384 antibody labeled with a green fluorescent tag in a multiple analyte analysis. The detection was shown to be specific for the peptide in question by competitive inhibition and was not affected by high levels of free biotin.

Antibody labeling: MAB 384 (Chemicon International, Inc., Temecula, CA) was labeled with Bodipy FL-X (Molecular Probes, Inc., Eugene, OR) using methods described by the manufacturer of the Bodipy succinymidyl ester. Absorbance at 280 nm and 504 nm revealed that the resulting Bodipy-labeled antibody had a Bodipy to protein ratio of 3.31 and was stored in PBS containing 1 mg/mL BSA as stabilizer.

Avidin conjugation to microspheres: Nine distinctly dyed DFM (5.5 μM, Bangs Laboratories, Inc. (Carmel, IN), dyed by Emerald Diagnostics, Inc. (Eugene, OR)) were conjugated separately to Neutravidin (deglycosylated avidin) with a two-step EDC coupling method (Pierce Chemicals, Rockford, IL) using sulfo-NHS to stabilize the amino-reactive intermediate. 20 μL (8.4 million microspheres) of each bead type was activated for 20 minutes in a total volume of 100 μL containing 500 μg of EDC and Sulfo-NHS in 50 mM sodium phosphate buffer, pH 7.0. The microspheres were washed twice with 100 μL PBS, pH 7.4 using centrifugation at 13,400 x g for 30 seconds to harvest the microspheres. Activated, washed beads were suspended in 50 μL of a 0.25 mg/mL solution of Neutravidin in PBS, pH 7.4. After 2 hours, the microspheres were blocked by addition of 50 μL of 0.2 M glycine, 0.02% Tween 20 in PBS, pH 7.4 and incubated for an additional 30 minutes. Protein coated microspheres were washed twice with 100 μL 0.02% Tween 20, 1 mg/mL BSA in PBS, pH 7.4 (PBSTB) and stored in PBSTB at approximately 3,000,000 microspheres/mL as determined by hemocytometer count.

Peptide attachment to microspheres: Each of the nine DFM conjugated to Neutravidin were treated separately with one of the nine biotinylated peptides. 10 μL of biotinylated peptides at 100 - 200 ng/mL was mixed with 10 μL of microspheres and reacted for 5 minutes followed by

2 x 100 µL washes in PBSTB. The peptide loaded microspheres were suspended in 20 µL of PBSTB.

Single analyte assay: 10 µL of each of the peptide loaded microspheres was reacted with 10 µL of the Bodipy-labeled MAB 384 at 15.5 µg/mL in PBSTB for 1 hour, diluted to 300 µL in PBSTB and assayed using flow cytometry. Negative controls included the microspheres without peptide and with the Bodipy MAB 384.

Multiple analyte assay: 10 µL of each of the 9 peptide loaded microspheres was mixed to produce a bead set. 10 µL of the set was reacted with 10 µL of the Bodipy-labeled MAB 384 at 15.5 µg/mL in PBSTB for 1 hour, diluted to 300 µL in PBSTB and assayed using flow cytometry. Negative controls included the microsphere set without peptide and treated with the Bodipy MAB 384.

Competitive inhibition with soluble peptide: 10 µL of each of the 9 peptide loaded microspheres was mixed to produce a bead set. 10 µL of the Bodipy-labeled MAB 384 at 15.5 µg/mL in PBSTB was reacted with 10 µL of soluble peptide containing the epitope sequence HYGSLPQK (SEQ ID NO. 1) at 10 µg/mL and incubated for 1 hr. The microsphere set was then treated with peptide absorbed Bodipy-labeled MAB 384 at 15.5 µg/mL for 1 hour, diluted to 300 µL in PBSTB and assayed using flow cytometry.

Examination of the effects of free biotin: 10 µL of each of the 9 peptide loaded microspheres was mixed to produce a bead set. 10 µL of the mixture was reacted with 10 µL of 10 µg/mL free biotin and incubated for 1 hr. The microsphere set was then treated with Bodipy-labeled MAB 384 at 15.5 µg/mL for 1 hour, diluted to 300 µL in PBSTB and assayed using flow cytometry.

Results

Description of peptides to be screened: The amino acid sequence upstream and downstream from the epitope of monoclonal antibody MAB 384 (amino acid 67-74, YGSLPQ, SEQ ID NO.

2) was determined using the published amino acid sequence (Roth, H.J., et al., J. Neurosci. Res.. *17*, 321-328, 1990). The table below shows the amino acid sequence of the nine overlapping peptides produced for the screening assay. Note that to the carboxy-terminal end of all peptides was added a glycine (G)-lysine (K)-biotin.

1	GLCNMYKDGK-biotin
2	MYKDSHHPGK-biotin
3	SHHPARTAGK-biotin
4	ARTAH**YGS**GK-biotin
5	H**YGSLPQ**KGK-biotin
6	**LPQ**KSHGRGK-biotin
7	SHGRTQDEGK-biotin
8	TQDENPVVGK-biotin
9	NPVVHFFKGK-biotin

Single vs. multiple analyte analysis: Each of the nine DFM coated with Neutravidin was reacted for 5 minutes with one of the nine biotinylated peptides diluted to 250 ng/mL in PBS. For single analyte analysis, each separate microsphere was reacted with Bodipy-labeled MAB 384 at 15.5 μg/mL for 60 minutes and the mixture assayed using flow cytometry. The Mean Intensity of Fluorescence (MIF) of the green fluorescence channel (F_m) is shown for each peptide-bead as the darker set of bars in **Figure 21**. The darkest bars represents single analyte analysis of each bead in the absence of peptide as a negative control.

For multiple analyte analysis, the nine bead-peptides were mixed and then reacted with Bodipy-MAB 384 at 15.5 μg/mL. After 60 minutes, the mixture was assayed using flow cytometry and results (MIF of F_m) are also shown in **Figure 21**. Both assays minus added peptide are shown as a negative control. Results indicated that peptide #5 contained the epitope for MAB 384. Peptides #4 and #6 although containing 3 of the epitope's amino acids showed little reactivity. The multiple and single analyte assays provided identical results. Numerical data is shown in **Table 6**.

Competitive inhibition using soluble epitope peptide: To further demonstrate the specificity of the assay, soluble peptide containing the epitope (#5) was used to inhibit the reaction shown in **Figure 21**. A 10 μL aliquot of the Bodipy-labeled MAB 384 was mixed with an equal volume of the epitope containing peptide (HYGSLPQK) at 10 μg/mL. After 1 hour the mixture was reacted with 10 μL of the bead mixture for 1 hour and assayed by flow cytometry. Results shown in **Figure 22** reveal that the reaction was significantly inhibited to a MIF of F_m of 53. Numerical data for the inhibition assay is shown in **Table 7**.

Effects of free biotin: The high avidity of the biotin-avidin interaction makes it unlikely that the various peptides could be released or exchanged from microsphere to microsphere, To demonstrate that such a release or exchange does not occur under strenuous conditions the following experiment was performed. A 10 μL aliquot of free biotin at 10 μg/mL (40 μM) was incubated with 10 μL of the bead-peptide mixture for 1 hour and then the microspheres reacted with the MAB 384 Bodipy at 15.5 μg/mL for 1 hour and assayed by flow cytometry. Results shown in **Figure 23** indicate that the free biotin at 10 μg/mL did not displace significant amounts of the biotinylated epitope peptide. Numerical data for the inhibition assay is shown in **Table 8**.

This epitope mapping example demonstrates the useful application of the instant invention to the area of combinatorial screening. The peptide carrying the epitope for the mouse monoclonal antibody screened in this example was clearly identified in a set of nine peptides. The identification was further shown to be specific by competitive inhibition with soluble epitope peptide. In addition, the stability of the avidin-biotin interaction for use with flow cytometry was demonstrated in an excess of free biotin.

TABLE 6

MIF of *Fm*

Bead	Peptide plus GL-Biotin	Assayed Single	Single no peptide	Assayed Multiple	Multiple no peptide
70/50	GLCNMYKD	72	66	28	28
60/70	MYKDSHHP	57	48	36	36
40/70	SHHPARTA	47	43	36	34
40/50	ARTAHYGS	57	47	35	27
70/70	HYGSLPQK	**1381**	66	**1348**	25
40/40	LPQKSHGR	43	44	67	25
40/60	SHGRTQDE	42	54	35	26
70/60	TQDENPVV	73	70	32	23
70/40	NPVVHFFK	60	60	29	21

TABLE 7

Peptide plus GL-Biotin	Assayed w/free Biotin
GLCNMYKD	4
MYKDSHHP	7
SHHPARTA	12
ARTAHYGS	13
HYGSLPQK	**53**
LPQKSHGR	15
SHGRTQDE	11
TQDENPVV	9
NPVVHFFK	17

TABLE 8

Bead	Peptide plus GL-Biotin	MIF Multiple	MIF w/ Biotin
70/50	GLCNMYKD	13	17
60/70	MYKDSHHP	17	19
40/70	SHHPARTA	20	22
40/50	ARTAH**YGS**	20	26
70/70	**HYGSLPQK**	**915**	**1023**
40/40	**LPQ**KSHGR	32	20
40/60	SHGRTQDE	19	23
70/60	TQDENPVV	31	34
70/40	NPVVHFFK	31	36

Multiple Analyte Simultaneous ToRCH Assay for Seroconversion.

This example demonstrates the utility of this invention in the screening of human serum for antibodies to infectious disease agents. Screening of serum for antibodies to certain infectious disease agents is often the only method available to determine if a patient has been, or is infected with the agent in question. For example, a common method of diagnosing HIV infection is by detection of HIV specific antibodies in the serum. This phenomenon known as seroconversion is commonly employed for diagnosis of several important pathogenic infections. One of the most commonly employed assay panels of this type is the ToRCH panel. ToRCH assays detect both serum IgG and serum IgM responses to *Toxoplasma gondii*, Rubella virus, Cytomegalovirus, and Herpes Simplex Virus Types 1 and 2. The importance of this assay especially to the pregnant woman has been well documented as any one of these infectious agents is capable of crossing the placental barrier and entering the immunologically naive fetus. These infectious agents can cause severe damage to the fetus and must be avoided. Currently , all ToRCH panel assays for antibodies specific to each of these pathogens is performed separately in a unique assay tube or microtiter well. This invention provides for a multiple analyte format that allows assay for either IgG or IgM antibodies specific for each of the five pathogens at the same time in the same tube with the same sample.

A ToRCH assay using flow cytometry has been developed by coupling purified antigens of *T. gondii,* Rubella, CMV and HSV Type 1 and Type 2 to five Differentially Fluorescent Microspheres (DFM). The specificity of the assay has been demonstrated by treating this bead set with human serum calibrators certified to be either positive or negative for all five agents. After this treatment, the bead set was treated with either Goat anti-human IgG-Bodipy or Goat anti-human IgM-Bodipy used to develop the assay. In addition, a third calibrator with known levels of reactivity to each agent was assayed and the results reported.

Antibody labeling: Goat anti-human IgG and goat anti-human IgM (Cappel Division, Organon Teknika, Durham, NC) were labeled with Bodipy FL-CASE (Molecular Probes, Inc., Eugene, OR) using methods described by the manufacturer of the Bodipy succinymidyl ester. Bodipy-labeled antibodies were stored in PBS containing 1 mg/mL BSA as stabilizer.

Antigen conjugation to microspheres: Five DFM (5.5 μM carboxylate, Bangs Laboratories, Inc., Carmel, IN, dyed by Emerald Diagnostics, Inc., Eugene, OR) were conjugated separately to the five ToRCH antigens (Viral Antigens, Inc.) with a two-step EDC coupling method (Pierce Chemicals, Rockford, IL) using sulfo-NHS to stabilize the amino-reactive intermediate. All antigens were dialyzed into PBS to remove any reactive amino groups such as sodium azide or glycine. The *T. gondii* preparation (Chemicon, Inc., Temecula, CA) was sonicated for 2 minutes in PBS, 10 mM EDTA to lyse the tachyzoites. 20 μL (8.4 million microspheres) of each bead type was activated for 20 minutes in a total volume of 100 μL containing 500 μg of EDC and Sulfo-NHS in 50 mM sodium phosphate buffer, pH 7.0. Microspheres were washed twice with 200 μL PBS, pH 7.4 using centrifugation at 13,400 x g for 30 seconds to harvest the microspheres. Activated and washed beads were suspended in 100 μL of antigen at 0.05 to 0.15 mg/mL in PBS, pH 7.4. After 2 hours, the microspheres were blocked by addition of 100 μL of 0.2 M glycine, 0.02% Tween 20 in PBS, pH 7.4 and incubated for an additional 30 minutes. Antigen coated microspheres were washed twice with 200 μL 0.02% Tween 20, 1 mg/mL BSA in PBS, pH 7.4 (PBSTB). and stored in PBSTB at approximately 3,000,000 microspheres/mL as determined by hemacytometer count.

Rubella assay: Rubella antigen loaded microspheres were used to examine several parameters of the assay in a single analyte format prior to the performance of multiple analyte assays. 10 μL (30,000 microspheres) of Rubella antigen coated beads were reacted with 10 μL of a 1:10 dilution of four different Rubella calibrator sera (Consolidated Technologies, Inc., Oak Brook, IL) and the mixture incubated for 1 hour. These sera were defined using a standard assay for the anti-Rubella IgG activity by the manufacturer of the calibrators. The units were defined as International Units/ mL or IU/mL. Beads were washed in PBSTB by centrifugation at 13,400 x g for 30 seconds and suspended in 40 μL of a 10 μg/mL solution of Bodipy-labeled anti-human IgG. This mixture was incubated for 1 hour, diluted to 300 μL in PBSTB and assayed using flow cytometry. Negative controls included the microspheres with no serum treated with the Bodipy-labeled antibodies. In addition one calibrator serum containing 70 IU/mL of anti-Rubella IgG activity was titrated in a single analyte assay.

Multiple analyte assay for IgG and IgM activities: Equivalent amounts of each of the 5 antigen loaded microspheres was mixed to produce a ToRCH bead mixture. 10 μL (30,000 microspheres) of the mixture was reacted with 10 μL of a 1:400 dilution of ToRCH control or calibrator sera and incubated for 1 hour. The positive and negative ToRCH control sera did not have defined units of activity. The ToRCH calibrator, however, did have defined levels of anti-ToRCH IgG activities as defined by INX and DiaMedix diagnostic instruments. These values were provided by the manufacturer for the lot of calibrator purchased. Beads were washed in PBSTB by centrifugation at 13,400 x g for 30 seconds and suspended in 20 μL of a 40 μg/mL solution of Bodipy-labeled anti-human IgG or IgM. This mixture was incubated for 1 hour, diluted to 300 μL in PBSTB and assayed using flow cytometry. Negative controls included the microspheres with no serum treatment and the microspheres treated with the ToRCH negative control serum. Both negative controls were developed with the Bodipy-labeled antibodies.

Results

Rubella assay: Rubella coated DFM were reacted with 4 human serum calibrators containing known levels of IgG antibodies specific for Rubella virions defined by International units

(IU/mL). The beads were washed and developed with goat anti-human IgG-Bodipy. Results are shown in **Table 9** and **Figure 24**. Increasing units of anti-Rubella activity were reflected in the Mean Intensity of Fluorescence (MIF) of F_m (green channel). Luminex Units (LU) were defined as the MIF of F_m for each data point minus the MIF of F_m for the negative control (no serum) multiplied by 0.1, and are included in **Table 9**.

Rubella calibrator titration: The human serum calibrator containing 70 IU/mL of anti-Rubella IgG was serially diluted in PBSTB and assayed with the Rubella coated microspheres and Bodipy-labeled anti-human IgG. Results shown in **Table 10** and **Figure 25** show that, as expected, the IgG antibodies specific for Rubella were titrated with dilution.

Multiple analyte ToRCH analysis for serum IgG and IgM: Each of the five distinct DFM coated with ToRCH antigens plus one DFM coated with human serum albumin (Miles, Inc., West Haven, CT) were mixed in equal volumes and 10 µL (30,000 microspheres) of the mixture reacted for 1 hour with triplicate, 20 µL aliquots of a 1:400 dilution of the ToRCH controls as well as the Low ToRCH calibrator. The calibrator from Blackhawk Systems, Inc. contained known levels of each pathogen specific antibody as measured on other diagnostic machines. After washing, one set of triplicates was developed with Bodipy-labeled anti-human IgG and another set with Bodipy-labeled anti-human IgM. Numerical results are shown in **Tables 11** and **12**. Results are presented graphically in **Figures 26A** and **26B**. Included in the figures are standard deviation bars for the triplicate measurements. For both IgG and IgM measurements, the ToRCH negative control serum (A96601, tubes #1-3) produced MIF of F_m similar to the negative control with no serum (tubes #10-12). The ToRCH positive control serum (A96602, tubes #4-6) demonstrated significant IgG activity to all five pathogens. Conversely, the positive control serum had only slight IgM based reactivity to the five pathogens. The known levels of anti-ToRCH IgG reactivities for the ToRCH Calibrator (A96500, tubes #7-9) were compared to the Luminex units of each IgG activity as determined by the multiple analyte analysis. Luminex units were defined by subtracting the negative control serum average MIF of F_m from the average MIF of F_m for each antigen and multiplying by 0.1. The levels of the ToRCH calibrator

were defined by the manufacturer as a factor of activity for each antigen above the limit of detection for that antigen on a specific diagnostic machine. These results are listed in **Table 13**.

A demonstrative ToRCH assay has been developed to simultaneously assay for serum IgG or IgM specific for the five ToRCH pathogens in a single tube. Results of the assay indicate that it is specific for each pathogen and is as sensitive as currently available instrument based assays. The multiple analyte format provides a uniquely powerful technology for rapid and less expensive serum testing for seroconversion to ToRCH pathogens as well as other infectious agents diagnosed in this manner.

TABLE 9: Anti-Rubella calibration curve

Calibrator IU/mL	MIF of *Fm*	LU/mL
360	1419	133
225	1004	91
70	458	37
40	376	28
0	92	0

TABLE 10: Anti-Rubella calibrator titration

70 IU/mL Calibrator Reciprocal of Dilution	MIF of *Fm*
1	4510
4	2554
8	1597
16	954
32	652
64	392
128	209
256	121
512	99
0	59

TABLE 11: IgG ToRCH assay

Tube #	Calibrator (1:400)	MIF of *Fm* in Triplicate					
		Toxo.	Rubella	CMV	HSV I	HSV II	HSA
1	A96601	21	9	12	16	17	22
2	A96601	22	8	10	17	15	26
3	A96601	25	9	11	14	17	22
4	A96602	647	1786	956	1223	664	78
5	A96602	590	1677	967	1511	719	81
6	A96602	620	1670	922	1348	611	72
7	A96500	103	38	50	128	64	27
8	A96500	95	43	48	127	58	43
9	A96500	87	41	49	127	56	29
10	No Serum	21	7	15	18	13	22
11	No Serum	23	8	11	15	19	19
12	No Serum	21	5	12	12	16	23
	Calibrator (1:400)	Average MIF of *Fm*					
		Toxo.	Rubella	CMV	HSV I	HSV II	HSA
	A96601	23	9	11	16	16	23
	A96602	619	1711	948	1361	665	77
	A96500	95	41	49	127	59	33
	No Serum	22	7	13	15	16	21

TABLE 12: IgM ToRCH assay

Tube #	Calibrator (1:400)	MIF of *Fm* in Triplicate					
		Toxo.	Rubella	CMV	HSV I	HSV II	HSA
1	A96601	40	10	17	17	21	16
2	A96601	36	9	15	15	20	17
3	A96601	39	9	19	18	23	20
4	A96602	68	109	53	80	52	27
5	A96602	69	112	56	84	52	23
6	A96602	77	133	81	91	64	60
7	A96500	66	15	27	34	26	20
8	A96500	67	15	23	37	29	22
9	A96500	66	15	28	31	31	29
10	No Serum	40	9	18	17	21	21
11	No Serum	36	8	20	17	19	16
12	No Serum	38	8	14	17	19	18
	Calibrator (1:400)	Average MIF of *Fm*					
		Toxo.	Rubella	CMV	HSV I	HSV II	HSA
	A96601	38	9	17	17	21	18
	A96602	71	118	63	85	56	37
	A96500	66	15	26	34	29	24
	No Serum	38	8	17	17	20	18

TABLE 13: Comparison of known levels of anti-ToRCH IgG for the ToRCH calibrator from Blackhawk BioSystems with Luminex Units

	T.gondii	Rubella	CMV	HSV 1	HSV 2
Diagnostic Machine used	INX	INX	INX	Diamedix	DiaMedix
Factor above Limit of Detection	1.7 x	2.7 x	1.7 x	2.5 x	1.1 x
Units of Activity	11.3 IU/mL	26.9 IU/mL	24.5 IU/mL	50 EU/mL	22 EU/mL
Luminex Units/mL	7.2 LU/mL	3.2 LU/mL	3.8 LU/mL	11.1 LU/mL	4.3 LU/mL

Simultaneous Assay of Dog Sera for Allergic IgE and Allergen-Specific IgG

This example demonstrates the screening of serum for IgE antibodies specific for allergens. Screening of serum for IgE antibodies specific to allergens is a viable option for allergy testing as compared with skin sensitivity testing. The instant invention provides for a format that can assay for either IgG or IgE responses to numerous allergens at the same time in the same tube with the same sample and is therefore a uniquely powerful method of screening.

An allergy assay has been developed including 16 grass allergens in a multiple analyte, simultaneous format. A panel of 16 grass allergens were attached to 16 Differentially Fluorescent Microspheres (DFM) with one grass allergen being coated onto one unique member of the bead set. The allergen bead set was treated with diluted dog serum for 1 hour and treated with a solution of either Goat anti-Dog IgE or goat anti-dog IgG-FITC for an additional hour. For the IgE assay, beads were washed clear of this antibody and the bead set treated with an affinity purified rabbit anti-goat IgG-FITC antibody as probe.

Results demonstrate a uniquely powerful method of serum screening for allergies that provides a true multiple analyte format, as well as sensitivity and specificity.

Allergen conjugation to microspheres: Sixteen DFM (5.5 μM carboxylate) were conjugated separately to 16 soluble grass allergens (provided by Dr. Bill Mandy, BioMedical Services, Austin, TX) with a two-step EDC coupling method (Pierce Chemicals, Rockford, IL) using sulfo-NHS to stabilize the amino-reactive intermediate. All grass allergens were diluted 1:100 into PBS, pH 7.4. 20 μL (8.4 million microspheres) of each bead type was activated for 20 minutes in a total volume of 100 μL containing 500 μg of EDC and Sulfo-NHS in 50 mM sodium phosphate buffer, pH 7.0. Microspheres were washed twice with 100 μL PBS, pH 7.4 using centrifugation at 13,400 x g for 30 seconds to harvest the microspheres. Activated, washed beads were suspended in 50 μL of diluted allergen. After 2 hours, the microspheres were blocked by addition of 50 μL of 0.2 M glycine, 0.02% Tween 20 in PBS, pH 7.4 and incubated for an additional 30 minutes. Protein coated microspheres were washed twice with

100 µL 0.02% Tween 20, 1 mg/mL BSA in PBS, pH 7.4 (PBSTB). and stored in PBSTB at approximately 3,000,000 microspheres/mL as determined by hemacytometer count.

Multiplexed K-9 grass allergen IgE assay: Equivalent amounts of each of the 16 grass allergen loaded microspheres was mixed to produce a bead mixture. 20 µL (60,000 microspheres) of the mixture was reacted with 60 µL of a 1:3 dilution of dog serum in PBSTB and the mixture incubated for 1 hour. Beads were washed in 200 µL PBSTB by centrifugation at 13,400 x g for 30 seconds and suspended in 40 µL of a 50 µg/mL solution of anti-dog IgE (provided by Dr. Bill Mandy, BioMedical Services, Austin, TX). After incubation for 1 hour, beads were washed in 200 µL PBSTB by centrifugation at 13,400 x g for 30 seconds. Beads were then treated with 40 µL of rabbit anti-goat IgG-FITC (Sigma, St. Louis, MO) at 20 µg/mL. After one hour the bead mixture was diluted to 300 µL in PBSTB and assayed using flow cytometry. Negative controls included the microspheres with dog serum, without the goat anti-dog IgE and with the rabbit anti-goat IgG-FITC. A negative control of the bead set with no dog serum was also included. Allergen specific dog IgE was determined by subtraction of the mean intensity of fluorescence (MIF) of the green channel (F_m) for the negative controls for each grass allergen from the MIF of F_m for the tubes including the goat anti-dog IgE.

Simultaneous K-9 grass allergen IgG assay: Equivalent amounts of each of the 16 grass allergen loaded microspheres was mixed to produce a bead mixture. 20 µL (8.4 million microspheres) of the mixture was reacted with 20 µL of a 1:10 dilution of dog serum in PBSTB and the mixture incubated for 1 hour. Beads were washed in 200 µL PBSTB by centrifugation at 13,400 x g for 30 seconds and suspended in 25 µL of a 50 µg/mL solution of goat anti-dog IgG-FITC. After one hour the bead mixture was diluted to 300 µL in PBSTB and assayed using flow cytometry. Negative controls included the microspheres with no dog serum and with the goat anti-dog IgG-FITC. Allergen specific dog IgG was determined by subtraction of the mean intensity of fluorescence (MIF) of the green channel (F_m) for the negative control for each grass allergen from the MIF of F_m for the tubes including dog serum.

Results

Multiple analyte dog anti-grass allergen IgG assay: Grass allergen coated DFM were reacted with 6 dog sera provided by BioMedical Services, Austin, TX that had been characterized by ELISA for anti-grass allergen IgE. The IgG response to these grass allergens was not measured by BioMedical Services. The beads were washed and developed with goat anti-dog IgG-FITC. Results are shown in **Figure 27**. The MIF of F_m in the absence of dog serum was subtracted from the MIF of F_m for each bead with each dog serum. Two dogs, A96324 and A96326 demonstrated relatively high IgG reactivity to most of the grass allergens. Two dogs, A96325 and A96317 demonstrated relatively medium IgG reactivity to most of the grass allergens. Two dogs, A96319 and A96323 demonstrated relatively low IgG reactivity to most of the grass allergens.

Multiple analyte dog anti-grass allergen IgE assay: Grass allergen coated DFM were reacted with 6 dog sera provided by BioMedical Services, Austin, TX that had been characterized by ELISA for anti-grass allergen IgE. The beads were washed and treated with goat anti-dog IgE for 1 hour. The assay was developed with rabbit anti-goat IgG-FITC. Results are shown in **Figure 28**. The MIF of F_m in the absence of dog serum was subtracted from the MIF of F_m for each bead with each dog serum. Two dogs, A96325 and A96326 demonstrated relatively low reactivity to most of the grass allergens with the exception of Wheat grass and several others for A96326. These results agree with the ELISA results provided by BioMedical Services. A96325 was negative for 11 grass allergens (only ones tested) and A96326 was negative for the same 11 grass allergens except for a "Borderline" result in ELISA against a mixture of Wheat and Quack grass (due to the non-multiplexed format of ELISA assays, allergens are often mixed to increase the throughput levels). The other four dog sera demonstrated medium to high IgE responses to several of the grass allergens. Although agreement between ELISA and flow cytometry assay results was not absolute, the two assays followed the same trends. Dogs with IgE reactivity to grass allergens were detected by both assays.

Comparison of multiple analyte IgG and IgE results: The IgG and IgE anti-grass allergen response to each of the 16 allergens was compared by graphing. **Figures 29-34** demonstrate that

there was no correlation between IgG and IgE response to grass allergens in the six dogs. Some dogs were low responders for both IgE and IgG, some were reactive with both immunoglobulin subclasses, and some demonstrated IgE reactivity in a low background of IgG specific for the grass allergens. Examination of the IgG reactivity in a serum could identify those sera in which the IgE reactivity could be masked by the high IgG reactivity.

A demonstrative assay for serum IgG or IgE activity to 16 grass allergens has been developed that allows simultaneous assay of all 16 allergens at the same time in the same tube using the same sample. Results with 6 dog sera suggested that IgE anti-grass allergen activity as determined by ELISA was in general agreement with results provided using flow cytometry. In addition, the ease of determination for IgG anti-grass allergen activity in the six dogs was demonstrated.

A Simultaneous Immunometric Assay For Human Chorionic Gonadotropin and Alpha-Fetoprotein

This example illustrates the determination of multiple analyte levels in a liquid sample simultaneously by immunometric or capture-sandwich assay. The use of capture-sandwich assays to accurately determine analyte levels in liquid solutions is a commonly used format for many analyte assays. The technique is especially useful for those analytes present in low quantities as the first step serves to capture and thus concentrate the analyte. The uniqueness of this assay is the multiple analyte format allowing the simultaneous determination of two distinct serum proteins at the same time in the same tube from the same serum sample.

Human chorionic gonadotropin (hCG), a gonadotropic hormone secreted by the placenta, is the primary hormonal marker utilized for pregnancy testing. hCG is elevated both in urine and serum during pregnancy. Alpha fetoprotein (AFP) is the fetal cell equivalent to human serum albumin. AFP is elevated in pregnancy and in certain types of malignancies. Many clinical fertility or pregnancy test panels include immunometric assays for these two serum proteins. Immunometric or capture-sandwich assays for hCG and AFP were developed separately and then combined in a multiple analyte format.

The hCG assay was developed by examining several antibody pairs for their ability to capture and quantitate hCG levels in solution. First, a monoclonal antibody was coupled through carbodiimide linkage to a carboxylate substituted Differentially Fluorescent Microsphere (DFM). Next, a polyclonal, affinity purified antibody was Bodipy-labeled and used to probe DFM captured hormone. Once this assay was adjusted to include physiological sensitive ranges, the process was repeated for AFP. Cross-reactivity of the two assays was examined to demonstrate that the two assays would not interfere. The assays were then performed simultaneously. Commercially available serum calibrators were used to demonstrate that both assays were sensitive in clinically relevant ranges and an unknown was include to demonstrate how the two assays work simultaneously.

Antibody labeling: The two affinity purified polyclonal anti-hCG (AB633) and anti-AFP (M20077) antibodies (Chemicon, Inc., Temecula, CA and Medix Division, Genzyme, San Carlos, CA) were labeled with Bodipy FL-CASE (Molecular Probes, Inc., Eugene, OR) using methods described by the manufacturer of the Bodipy succinymidyl ester. The resulting Bodipy-labeled antibodies were stored in PBS containing 1 mg/mL BSA as stabilizer.

Antibody conjugation to microspheres: Monoclonal anti-hCG (MAB602) and anti-AFP (S10473) capture antibodies were conjugated to microspheres with a two-step EDC coupling method (Pierce Chemicals, Rockford, IL) using sulfo-NHS to stabilize the amino-reactive intermediate. 20 µL (8.4 million microspheres) of each DFM was activated for 20 minutes in a total volume of 100 µL containing 500 µg of EDC and Sulfo-NHS in 50 mM sodium phosphate buffer, pH 7.0. Microspheres were washed twice with 200 µL PBS, pH 7.4 using centrifugation at 13,400 x g for 30 seconds to harvest the microspheres. Washed, activated beads were suspended in 50 µL of a 0.05 mg/mL solution of antibody in PBS, pH 7.4. After 2 hours, microspheres were blocked by addition of 50 µL of 0.5 mg/mL BSA, 0.02% Tween 20 in PBS, pH 7.4 and incubated for an additional 30 minutes. Protein coated microspheres were washed twice with 200 µL 0.02% Tween 20, 1 mg/mL BSA in PBS, pH 7.4 (PBSTB) and stored in PBSTB at approximately 3,000,000 microspheres/mL. Microsphere concentrations were determined using a hemacytometer.

Antibody pairs analysis of hormone capture assay: Capture assay antibody pairs were screened by coupling potential capture antibodies to microspheres and assaying them using all potential combinations of capture antibody-bead/ Bodipy-labeled probe antibody. Assays were performed using 10 μL of capture antibody microspheres (approximately 30,000) plus 20 μL of antigen solution at 10 μg/mL in PBSTB for a 1 hour incubation. Beads were washed in PBSTB by centrifugation at 13,400 x g for 30 seconds and suspended in 20 μL of a 25 μg/mL solution of Bodipy-labeled probe antibody. This mixture was incubated for 1 hour, diluted to 300 μL in PBSTB and assayed using flow cytometry.

Antigen titration assay: Once an antibody pair was chosen for use, the pair was analyzed for sensitivity and limit of detection by titration of antigen. Assays were performed using 10 μL of capture antibody microspheres plus 20 μL of antigen dilutions in PBSTB for a 1 hour incubation. Beads were washed in 200 μL PBSTB by centrifugation at 13,400 x g for 30 seconds and suspended in 20 μL of a 25 μg/mL solution of Bodipy-labeled probe antibody. This mixture was incubated for 1 hour, diluted to 300 μL in PBSTB and assayed using flow cytometry.

Cross-reactivity analysis: To examine the possibility of cross-reactivity, 10 μL of MAB 602 anti-hCG capture beads (5,000 microspheres) were treated with 20 μL dilutions of hCG or AFP. After 1 hour the beads were washed in 200 μL PBSTB by centrifugation at 13,400 x g for 30 seconds and suspended in 20 μL of either Bodipy-labeled anti-hCG or Bodipy-labeled anti-AFP at 25 μg/mL. Conversely, 10 μL of S-10473 anti-AFP capture beads (5,000 microspheres) were treated with 20 μL dilutions of hCG or AFP. After 1 hour, beads were washed in 200 μL PBSTB by centrifugation at 13,400 x g for 30 seconds and suspended in 20 μL of either Bodipy-labeled anti-hCG or Bodipy-labeled anti-AFP at 25 μg/mL. Mixtures were incubated for 1 hour, diluted to 300 μL in PBSTB and assayed using flow cytometry.

Washed vs. no-wash assay format: An AFP/hCG capture antibody bead mixture was made by mixing equal amounts of the two bead types. In duplicate, 10 μL of this bead mixture (10,000 microspheres) was mixed with 20 μL dilutions of AFP/ hCG and incubated for 1 hour. One set

of beads were washed in PBSTB by centrifugation at 13,400 x g for 30 seconds and suspended in 20 µL of a mixture of Bodipy-labeled anti-hCG and anti-AFP both at 25 µg/mL. This mixture was incubated for 1 hour, diluted to 300 µL in PBSTB and assayed using flow cytometry. The second set of beads were treated directly with 20 µL of a mixture of Bodipy-labeled anti-hCG and anti-AFP both at 25 µg/mL. This mixture representing a homogenous (no-wash) assay was also incubated for 1 hour, diluted to 300 µL in PBSTB and assayed using flow cytometry.

Multiple analyte assay: Once the AFP and hCG antibody pairs were shown not to cross-react and were adjusted to provide clinically relevant ranges of sensitivity in a homogenous assay, the assays were performed simultaneously using commercially available serum calibrators as the source of AFP and hCG antigens. Equivalent amounts of each of the two capture antibody loaded microspheres was mixed to produce an AFP/hCG capture mixture. In triplicate, 10 µL of this bead mixture (5,000 of each microsphere) was mixed with 20 µL of three serum calibrators (high, medium and low) containing known levels of AFP and hCG and incubated for 1 hour. Mixtures were treated directly with 20 µL of a blend of Bodipy-labeled anti-hCG and anti-AFP both at 25 µg/mL. Mixtures were incubated for 1 hour, diluted to 300 µL in PBSTB and assayed by flow cytometry.

Results

Antibody pair analysis for hCG capture assay: For hCG antibody pair analysis, five capture antibody/microspheres were prepared and the identical five antibodies were Bodipy-labeled to serve as probes. Three of the antibodies were specific for the alpha sub-unit of hCG and two for the beta sub-unit. The three anti-alpha sub-unit antibody/microspheres were assayed for utility with the two Bodipy-labeled anti-beta hCG antibodies. Conversely, the two anti-beta sub-unit antibody/microspheres were assayed for utility with the three Bodipy-labeled anti-alpha hCG antibodies. Results of this screen are shown in **Table 14** and **Figure 35**. The 12 combinations of antibodies are shown with (odd numbers) and without (even numbers) hCG at 20 µg/mL. It is apparent that the first two antibody pairs, #1 and #3 demonstrated the highest mean intensity of fluorescence (MIF) of the F_m (green channel). Further examination of these two pairs led to

the decision to chose the #3 pair of MAB 602 for capture antibody and AB633-Bodipy as probe antibody for the hCG capture/sandwich assay.

Antigen titration: The MAB 602/AB633 anti-hCG capture system was assayed by hCG titration to determine if the level of sensitivity required for clinical assay was achievable. A limit of detection of at least 1 ng hCG/mL was the target as this was the level of hCG in the low serum calibrator to be used later in this project. The results of this antigen titration is shown in **Table 15** and **Figure 36**. The limit of detection was between 20 and 200 pg/mL. This revealed that the MAB602/AB633 anti-hCG antibody pair was sufficiently sensitive for hCG analysis. Included in this analysis was MIF of F_m measurements from counting of 100 or 1000 microspheres. Results were similar. A similar analysis of antibody pairs and antigen titration for AFP identified an AFP pair that could be further developed.

Cross-reactivity assay: The MAB 602/AB633 anti-hCG capture system and S-10473/M20077 anti-AFP capture system were examined for cross reactivity by assaying each capture bead with each antigen and Bodipy-labeled antibody. Results are shown in **Table 16** and **Figures 37A** and **37B**. No significant cross-reactivity between the hCG and AFP capture systems was detected.

No-wash vs. washed assay format: The hCG and AFP assays were performed simultaneously and examined for the limit of quantitation or dynamic range in both a washed format and no wash or homogenous format. Result of these antigen titrations are shown in **Table 17** and **Figures 38A** and **38B**. Results indicated that the homogenous format provided sufficient dynamic range for the purposes of clinical relevance.

Multiple analyte hCG/AFP assay: The two assays were performed simultaneously using serum calibrators of known hCG and AFP levels to generate a standard curve. For each standard curve one serum of unknown hCG and AFP level was included to demonstrate how the assay would determine the level of hCG and AFP in the serum.

The Randox Tri-level calibrators consisted of three serum samples with high, medium and low levels of hCG and AFP documented in mU or U/mL for hCG and AFP respectively. These calibrators are used in at least 12 different diagnostic instruments including those of Abbott (Abbott Park, IL), bioMerieux (St. Louis, MO), Ciba Corning (Medfield, MA), Diagnostics Products (Los Angeles, CA), Kodak (Rochester, NY), Syva (San Jose, CA), Tosoh (Atlanta, GA) and Wallac (Gaithersburg, MD). Literature with the Randox Tri-Level control listed the units of each known analyte as defined by each diagnostic machine. We calculated the average of the hCG mU/mL and AFP U/mL for the three calibrators. In the case of the hCG, the low and medium calibrators contained 22.8 and 26.4 mU/mL which were extremely close considering the distance to the high calibrator (436 mU/mL). For this reason, we included a 1:2 dilution of the high range calibrator into hCG/AFP certified negative serum to produce a fourth level serum calibrator termed Level 3D. Calibrator 3D was only used in construction of the hCG standard curve so each of the assays was effectively defined by three point calibration.

Table 18 shows the results of this multiple analyte assay. The assay was performed in triplicate and the average MIF of F_m computed for graphing. Coefficients of variation (CV) for the triplicates were consistently less than 10% are shown. Also included in the table are the number of microspheres correctly identified by the flow cytometry out of the 400 counted per tube. Of the 400 beads counted the expected ratio of MAB 602 containing 60/40 beads to S-10473 containing 40/60 beads was 1:1. Therefore of the 200 beads expected, this was the number of beads correctly identified and used to compute the MIF of F_m for that data point.

Figures 39A and **39B** graphically represent the data of **Table 18**. For both hCG and AFP a plot of the MIF vs. the log of antigen concentration produced a line that was best fit using a third level polynomial equation. The fit for the hCG curve provided an R^2 of 1.0 and for AFP an R^2 of 0.9999 was achieved. Using the polynomial equation, the concentration of the unknowns was computed. Results of these analyses are seen in **Table 18**. The unknown serum contained 218.55 ± 6.56 mU/mL of hCG and 39.59 ± 1.19 U/mL of AFP.

A demonstrative immunometric assay for hCG and AFP in serum has been developed. Assays were first developed as single analyte or single bead assays, and optimized with regards to sensitivity, limit of quantitation and cross-reactivity. The assays were then combined to quantitatively determine multiple analyte levels in a liquid solution in the same tube from the same sample at the same time. Results, using commercially available calibrator sera, has proven that this invention is effective for this type of quantitative assay.

TABLE 14

Sample	Description	hCG Conc. (μg/mL)	MIF of *Fm*
1	A1-Beads + B1 Ab-BD with hCG	20.0	8790
2	A1-Beads + B1 Ab-BD with no hCG	0.0	108
3	A2-Beads + B1 Ab-BD with hCG	20.0	9441
4	A2-Beads + B1 Ab-BD with no hCG	0.0	163
5	A3-Beads + B1 Ab-BD with hCG	20.0	3150
6	A3-Beads + B1 Ab-BD with no hCG	0.0	2984
7	A1-Beads + B2 Ab-BD with hCG	20.0	2287
8	A1-Beads + B2 Ab-BD with no hCG	0.0	37
9	A2-Beads + B2 Ab-BD with hCG	20.0	1232
10	A2-Beads + B2 Ab-BD with no hCG	0.0	42
11	A3-Beads + B2 Ab-BD with hCG	20.0	566
12	A3-Beads + B2 Ab-BD with no hCG	0.0	560
13	B1-Beads + A1 Ab-BD with hCG	20.0	70
14	B1-Beads + A1 Ab-BD with no hCG	0.0	23
15	B2-Beads + A1 Ab-BD with hCG	20.0	346
16	B2-Beads + A1 Ab-BD with no hCG	0.0	20
17	B1-Beads + A2 Ab-BD with hCG	20.0	107
18	B1-Beads + A2 Ab-BD with no hCG	0.0	33
19	B2-Beads + A2 Ab-BD with hCG	20.0	886
20	B2-Beads + A2 Ab-BD with no hCG	0.0	56
21	B1-Beads + A3 Ab-BD with hCG	20.0	105
22	B1-Beads + A3 Ab-BD with no hCG	0.0	196
23	B2-Beads + A3 Ab-BD with hCG	20.0	143
24	B2-Beads + A3 Ab-BD with no hCG	0.0	609

TABLE 15

Sample	hCG Conc. (ng/mL)	MIF of *Fm* (1000 Beads)	MIF of *Fm* (1000 Beads)
1	20000	9337	9222
2	2000	9286	9392
3	200	9233	9400
4	20	8497	8664
5	2	1286	1382
6	0.2	258	254
7	0.02	120	147
8	0.002	122	121
9	0.0002	122	149
10	0	128	111

TABLE 16A

MAB602 BEADS -		**Anti-hCG**			
Samp.	**Antigen** ng/mL	hCG anti-hCG	AFP anti-hCG	hCG anti-AFP	AFP anti-AFP
1	1000.0	792	53	47	52
2	100.0	761	47	48	48
3	10.0	530	47	47	48
4	1.0	104	47	48	48
5	0.1	55	52	49	48
6	0.0	48	71	72	48

TABLE 16B

M20077 BEADS -		**Anti-AFP**			
Samp.	**Antigen** ng/mL	hCG anti-hCG	AFP anti-hCG	hCG anti-AFP	AFP anti-AFP
1	1000.0	99	57	78	348
2	100.0	54	75	44	356
3	10.0	44	44	45	103
4	1.0	51	50	44	98
5	0.1	42	75	49	44
6	0.0	43	61	45	45

TABLE 17

	AFP				hCG		
Sample No.	AFP ng/mL	No Wash	Washed		hCG ng/mL	No Wash	Washed
1	1000	379	1481		2000	491	3194
2	500	643	1376		1000	770	3158
3	250	956	1205		500	1198	3342
4	125	1063	1052		250	1521	2755
5	62	980	814		125	2068	2949
6	31	639	612		62	2417	3200
7	16	359	347		31	2514	3183
8	8	190	205		16	2440	2528
9	4	94	108		8	1761	1955
10	2	51	59		4	1122	1300
11	1	33	35		2	650	547
12	0.5	24	25		1	330	359
13	0.25	17	24		0.5	166	175
14	0	15	13		0	15	18

TABLE 18

		hCG capture system					AFP capture system				
Tube No.	Descript.	hCG mU/mL	AFP U/mL	MIF of FL1	MIF AVG	MIF CV%	Beads IDed	MIF of FL1	MIF AVG	MIF CV%	Beads IDed
1	Level 1			23			114	74			98
2	Level 1	22.8	10.7	27	25.67	7%	130	83	78.00	5%	80
3	Level 1			27			140	77			72
4	Level 2			32			93	351			58
5	Level 2	26.4	53.8	34	31.67	6%	85	365	362.67	2%	61
6	Level 2			29			94	372			65
7	Level 3D			268			92	535			56
8	Level 3D	218	111.5	276	271.67	1%	101	562	552.00	2%	69
9	Level 3D			271			96	559			61
10	Level 3			631			106	1109			46
11	Level 3	436	223	601	624.00	3%	99	994	1061.00	5%	38
12	Level 3			640			97	1080			40
13	Negative			8			99	11			104
14	Negative	10	2	7	7.33	6%	111	13	12.33	8%	106
15	Negative			7			119	13			95
16	Unknown			270			140	268			67
17	Unknown	218.55	39.59	264	272.33	3%	141	274	276.00	3%	81
18	Unknown			283			113	286			84

Multiplexed Beadset Standard Curve Using an Inhibition Assay

This example provides a demonstration of the measurement of ligand-ligate reactions using a multiplexed beadset standard curve. Commonly for ligand-ligate reactions quantitation, known amounts of the ligand or ligate are introduced to the reaction leading to the production of a standard curve. Values for unknown samples are compared to the standard curve and quantified. The true multiple assay capability of this invention allows for an additional type of standard to be utilized. A multiplexed beadset standard curve for measuring analyte concentration is created by using several Differentially Fluorescent Microspheres (DFM) coated with either 1) different amounts of ligand (antigen), or 2) different amounts of ligate (antibody), or 3) different ligates possessing different avidities for the ligand (different monoclonal antibodies). We have demonstrated an example of the first type of multiple analyte standard curve by developing a competitive inhibition assay for human IgG.

Four DFM were coated with human IgG at four different concentrations. When probed with goat anti-human IgG-Bodipy the Mean Intensity of Fluorescence (MIF) of F_m (green channel) for each bead subset was different. The MIF of F_m correlated with the amount of hIgG used to coat the beads in each subset. If soluble hIgG was mixed with the reaction in a competitive manner the MIF of F_m was reduced for each bead as less of the probe antibody was bound to the beads. In a normal standard curve, the signal (MIF of F_m) is plotted against the concentration of the inhibitor. For the multiplexed beadset standard curve, the slope of the MIF of F_m across the beads within a subset is plotted against the concentration of inhibitor. Comparison of the two types of standard curves revealed them to be of equivalent value for prediction of an unknown amount of inhibitor.

Human IgG conjugation to microspheres: Four DFM (5.5 μM carboxylate, Bangs Laboratories, Inc., Carmel, IN, dyed by Emerald Diagnostics, Inc., Eugene, OR) were conjugated separately to 4 different concentrations of hIgG (Cappel Division, Organon Teknika, Durham, NC) with a two-step EDC coupling method (Pierce Chemicals, Rockford, IL) using sulfo-NHS to stabilize the amino-reactive intermediate. 20 μL (8.4 million microspheres) of each bead type was activated for 20 minutes in a total volume of 100 μL containing 500 μg of EDC and Sulfo-NHS

in 50 mM sodium phosphate buffer, pH 7.0. The microspheres were washed twice with 200 µL PBS, pH 7.4 using centrifugation at 13,400 x g for 30 seconds to harvest the microspheres. Activated, washed beads were suspended in 50 µL of hIgG at 50, 10, 5, and 1 µg/mL in PBS, pH 7.4. After 2 hours, the microspheres were blocked by addition of 50 µL of 0.2 M glycine, 0.02% Tween 20 in PBS, pH 7.4 and incubated for an additional 30 minutes. Protein coated microspheres were washed twice with 200 µL 0.02% Tween 20, 1 mg/mL BSA in PBS, pH 7.4 (PBSTB). and stored in PBSTB at approximately 3,000,000 microspheres/mL as determined by hemacytometer count.

Antibody labeling: Goat anti-human IgG (Cappel Division, Organon Teknika, Durham, NC) was labeled with Bodipy FL-CASE (Molecular Probes, Inc., Eugene, OR) using methods described by the manufacturer of the Bodipy succinymidyl ester. The resulting Bodipy-labeled antibody was stored in PBS containing 1 mg/mL BSA as stabilizer.

Multiplexed beadset standard curve: Equivalent amounts of each of the 4 differentially loaded IgG microspheres was mixed to produce a bead mixture. 10 µL of the goat anti-hIgG-Bodipy at 25 µg/mL in PBSTB was mixed with 10 µL of a dilution of hIgG in PBSTB. Immediately 10 µL (30,000 microspheres) of the bead mixture was added and the mixture incubated at room temperature for 30 minutes. Beads were diluted to 300 µL in PBSTB and assayed using flow cytometry. A negative control included the microspheres with the goat anti-hIgG-Bodipy with no inhibitor (hIgG). Each bead subset was assigned the value of a consecutive integer (i.e. the bead subset coupled with the lowest concentration of IgG was given a value of 1, the next highest concentration was given a value of 2, etcetera) and those numbers plotted against the MIF of each bead subset at each concentration of inhibitor tested. The slopes (designated here as inter-bead subset slopes) were computed using linear regression analysis. The inter-subset slopes were then plotted against the concentration of inhibitor using a logarithmic scale for the concentration of inhibitor. Results were plotted as the slope of the MIF of F_m across the bead set against the log of hIgG concentration. Curve fitting was performed using a power function trendline and the R^2 correlation was reported. For a perfect fit, R^2=1.0.

Common standard curve: Using the data from the assay described above, a common standard curve was constructed to compare results with the multiple analyte standard curve. Data from the DFM coated at 50 μg/mL hIgG was utilized to produce a five-point standard curve by plotting the MIF of F_m against the log of hIgG concentration. Curve fitting was performed using a power function trendline and the R^2 correlation was reported.

Results

Multiplexed beadset standard curve for a competitive inhibition assay: Four differentially loaded IgG microspheres were utilized in a multiple beadset competitive inhibition assay for hIgG at five different concentrations of soluble inhibitor (hIgG). Results of the assay are shown in **Table 19**. The inhibition pattern on each bead is plotted in **Figure 40**. The inter-bead subset slopes are plotted against the log concentration of inhibitor in **Figure 41**. A Power Trendline in Excel was used to produce the R^2 of 0.9933.

Common standard curve using one bead of the multiple analyte assay: Data from the 50 μg/mL hIgG bead was utilized to produce a five-point standard curve by plotting the MIF of F_m against the log of hIgG concentration. Results are shown in **Figure 42**. Curve fitting was performed using a Power function trendline and $R^2 = 0.9942$.

A novel type of standard curve for ligand-ligate measurement was demonstrated. Results suggested that the multiplexed beadset standard curve was of similar utility as the common multi-point standard curve in quantitation of unknown samples. Advantages of the multiplexed beadset standard curve include the inclusion of the standard curve microspheres in each point of a multiplexed beadset assay, and the extension of an assay's dynamic range. This may be achieved by increasing the concentration range of ligand or ligate on the microspheres or by increasing the range of avidities for ligand on a series of microspheres.

TABLE 19

Samp	Inhibitor Conc (μg/mL)	Bead 1 1.0 μg/mL IgG	Bead 2 5 μg/mL IgG	Bead 3 10 μg/mL IgG	Bead 4 50 μg/mL IgG	SLOPE
1	100	14	77	108	288	85.3
2	50	21	100	162	428	128.3
3	25	40	166	267	844	251.3
4	12.5	110	463	747	1467	435.5
5	6.25	257	1226	1629	2316	658
6	0	134	793	1432	2217	688.8

Nucleic Acid Measurement

The power and sensitivity of PCR has prompted its application to a wide variety of analytical problems in which detection of DNA or RNA sequences is required. One major difficulty with the PCR technique is the cumbersome nature of the methods of measuring the reaction's products - amplified DNA.

A major advance in this area is here. That advance employs a flow cytometric bead-based hybridization assay which permits the extremely rapid and accurate detection of genetic sequences of interest. In a preferred embodiment of that invention, a bead to which a nucleic acid segment of interest has been coupled is provided. A PCR product of interest (or any other DNA or cDNA segment) is detected by virtue of its ability to competitively inhibit hybridization between the nucleic acid segment on the bead and a complementary fluorescent nucleic acid probe. The method is so sensitive and precise as to allow the detection of single point mutations in the PCR product or nucleic acid of interest. Although that method in itself provides a pivotal advance in the art of analyzing PCR reaction products, the further discovery of methods of multiplexing such an analysis, compounds the method's power and versatility to allow simultaneously analysis of a number of nucleic acid products or a number of sequences within a single product in a single sample.

The multiplexed DNA analysis method described here can be applied to detect any PCR product or other DNA of interest for specific polymorphisms or mutations or for levels of expression, e.g. mRNA. With the multiplexed techniques provided by the instant invention, individuals can be screened for the presence of histocompatibility alleles associated with susceptibility to diseases, mutations associated with genetic diseases, autoimmune diseases, or mutations of oncogenes associated with neoplasia or risk of neoplasia. The analysis of DNA sequences occurs generally as follows:

1. A beadset containing subsets of beads coupled to nucleic acid sequences of interest is prepared by coupling a unique synthetic or purified DNA sequence to the beads within each subset.
2. Fluorescent probes complementary to the DNA coupled to each bead subset are prepared. Methods known in the art, e.g., as described in U.S. Patent No. 5,403,711, issued April 4, 1995 and incorporated herein by reference, or other methods may be used to fluorescently label the DNA. Since each probe will bind optimally only to its complementary DNA-containing subset, under the conditions of the assay, the fluorescent probes may be added to the subsets before or after the subsets are pooled, and before or after addition of the DNA test sample(s) of interest.
3. Tissue, fluid or other material to be analyzed is obtained, and DNA is purified and/or amplified with PCR as necessary to generate the DNA products to be tested.
4. The DNA samples of interest are then mixed with the pooled beadset under suitable conditions to allow competitive hybridization between the fluorescent probes and the DNA of interest.
5. The beadset is then analyzed by flow cytometry to determine the reactivity of each bead subset with the DNA sample(s). If the test sample contains a DNA sequence complementary to the DNA of a given bead subset then that subset will exhibit a decreased F_m value relative to the F_m value of beads to which a control DNA has been added. A computer executed method in accordance with the current invention can determine the subset from which each bead is derived, and therefore, the identity of the DNA sequence on the bead and any change in F_m.

Detection of Foreign DNA

The methods of the present invention find wide utility in the detection of foreign DNA's in, for example, diagnostic assays. Although the DNA segment to be analyzed can be any DNA sequence, in accordance with this embodiment the selected segment will be a DNA segment of a pathogenic organism such as, but not limited to, bacterial, viral, fungal, mycoplasmal, rickettsial, chlamydial, or protozoal pathogens. The procedure has particular value in detecting infection by pathogens that are latent in the host, found in small amounts, do not induce inflammatory or immune responses, or are difficult or cumbersome to cultivate in the laboratory. The multiplexed DNA detection method of the present invention is likely to find particular utility as a diagnostic assay for analysis of a sample from a patient having clinical symptoms known to be caused by a variety of organisms using a beadset designed to detect DNAs from the variety of organisms known to cause such symptoms to determine which of such organisms is responsible for the symptoms. DNA would be extracted from tissue, fluid or other sources and analyzed as described above.

Analysis of Genetic Polymorphisms

The invention may also be used to measure a variety of genetic polymorphisms in a target DNA of interest. For example, there are several genes in the MHC and many are polymorphic. There are at least two applications in which determination of the alleles at each position of the MHC is of critical importance. The first is the determination of haplotype for transplantation, and the second is determination of haplotype as indicator of susceptibility to disease. See Gross et al., "The Major Histocompatibility Complex-Specific Prolongation of Murine Skin and Cardiac Allograft Survival After In Vivo Depletion of $V\beta^+$ T Cells," J. Exp. Med., 177, 35-44 (1993). The MHC complex contains two kinds of polymorphic molecules, Class I genes, HLA A, B and D which have 41, 61 and 18 known alleles and Class 10 genes, HLA-DRI,3,4,5 HLA-DQAI and BI HLA-DP, DPA1, DPB1, also with many alleles. Each human can have up to 6 co-dominant Class I genes and 12 co-dominant Class 10 genes.

In the case of transplantation, the closer the match between the donor and recipient the greater the chance of transplant acceptance. A multiplexed assay in accordance with the

invention may be employed to perform tissue typing quickly and accurately to identify suitable matches for transplantation.

In the situation of disease association, it has been found that individuals bearing certain alleles are more prone to some diseases than the remainder of the population. The frequency of alleles of the MHC genes is not equal, and sets of alleles are frequently found (linkage disequilibrium) so that the identification of the exact set of alleles associated with many diseases is feasible. As one example, insulin-dependent diabetes mellitus (IDDM) is associated with certain HLA-DQ alleles. The number of alleles of DQ in the population is modest and genetic typing by PCR amplification and hybridization with allele specific probes has been shown to be practical. See Saiki et al., "Genetic Analysis of Amplified DNA with Immobilized Sequence-Specific Oligonucleotide Probes," Proc. Natl. Acad. Sci. U.S.A., 86, 6230- 6234 (1989).

For an assay of MHC in accordance with the invention, DNA is obtained from blood or other extractable source, and amplified with primers specific for the MHC genes under analysis, for example, HLA-DQA. For a full genotyping of the MHC, several samples of DNA would be amplified with different sets of primers to accommodate the large number of loci and the high degree of polymorphism. The PCR products are then screened for specific alleles using beadsets and fluorescent probes as described above.

Mutation Analysis of Selected Genes: Screening Procedures

There are several methodologies for determining and comparing DNA sequences in order to detect mutations which are associated with disease or neoplasia. When adapted to a bead-based, multiplexed format in accordance with the current invention, hybridization analysis allows for the rapid screening of multiple genetic loci for multiple wild type and mutant sequences.

In a preferred embodiment of the invention, a given genetic locus, or multiple loci, can be simultaneously screened for the presence of wild type or mutant sequences. In the same analysis, multiple known mutations can be distinguished from each other and from the wild type

sequence and uncharacterized mutations. In addition, the homozygosity or heterozygosity of known sequences can be determined.

A general approach for detecting a DNA mutation in accordance with this aspect of the invention is as follows. In a first step, a suitable probe for detecting a mutation of interest is selected. In an illustrative embodiment, selected oligonucleotides, representing wild-type and mutant sequences, from a region of a gene known to contain a mutation are prepared. Such oligonucleotides are coupled to microspheres by techniques known in the art, (e.g., carbodiimide coupling, or other means) to produce individual aliquots of beads having known oligonucleotides coupled thereto. The oligonucleotides must be a sufficient length to allow specific hybridization in the assay, e.g., generally between about 10 and 50 nucleotides, more preferably between about 20 and 30 nucleotides in length. In a preferred embodiment, a saturating amount of the oligonucleotide is bound to the bead. Fluorescent oligonucleotides, complementary to all or part of the sequences attached to each bead, are also prepared.

Next, PCR primers are selected to amplify that region of the test DNA corresponding to the selected probe, which are then used to amplify the particular region of DNA in the sample that contains the sequence corresponding to the oligonucleotide coupled to the beads. Either double stranded or single stranded PCR techniques may be used. If double stranded product is produced, the amplified PCR product is made single stranded by heating to a sufficient temperature to and for a sufficient time to denature the DNA (e.g., for about 1 to about 5 minutes at about 90-95°C in 2.3X SSC hybridization buffer). The mixture is cooled, and the beads are added and incubated with the PCR product under conditions suitable to allow hybridization to occur between the oligonucleotide on the beads and the PCR product (e.g., at room temperature for about 10 minutes). The fluorescent DNA probe may then be added and the entire mixture incubated under hybridization conditions suitable to allow competitive hybridization to occur (e.g., 5 minutes at 65°C, then cooling to room temperature over a period of several hours in 2.3X SSC buffer). As those of skill in the art will recognize, the concentrations of the PCR product and fluorescent probe to be used may vary and may be adjusted to optimize the reaction.

In general, the concentrations of PCR product and fluorescent probe to be used are adjusted so as to optimize the detectable loss of fluorescence resulting from competitive inhibition without sacrificing the ability of the assay to discriminate between perfect complementarity and one or more nucleotide mismatches. In an exemplary assay, the concentration of PCR product complementary to the oligonucleotide bound to the beads may be on the order of 1 to 10 times the molar concentration of fluorescent probe used. The fluorescent probe should preferably be added in an amount sufficient to achieve slightly less than saturation of the complementary oligonucleotide on the beads in order to obtain maximum sensitivity for competitive inhibition.

In a multiplexed assay employing the above principles, beadsets are separately prepared, pooled, and the bead-based hybridization analysis performed. In order to screen a given locus for mutations, beadset subsets are prepared such that subset 1 is coupled to a DNA segment identical to the wild type sequence, subset 2 is coupled to a DNA segment identical to a known mutation 1 (which may represent a single or multiple point mutations, deletions or insertions), subset 3 is coupled to a DNA segment identical to a second known mutation 2, and so on. The subsets are then mixed to create a pooled beadset.

When a nucleic acid sample is analyzed with such a beadset, only the bead subsets containing sequences identical to the test sample will show a large decrease in fluorescence (F_m). Bead subsets containing unrelated or greatly disparate sequences will show little or no decrease in fluorescence (F_m) and bead subsets containing very closely related sequences, such as point mutants, will show an intermediate decrease in fluorescence (F_m). Thus, a large decrease in the F_m of only subset 1 would indicate homozygous wild-type; a large decrease in the F_m of both subset 1 and subset 2 would indicate heterozygous wild-type / mutant 1 and so on. If the test sample is less inhibitory than the perfectly complementary sequence for any of the known sequences represented by the subsets then a new uncharacterized mutation is indicated. The test sample could then be sequenced to characterize the new mutation, and this sequence information used to construct a new subset for the beadset to detect the newly discovered mutation.

The present invention has wide-spread advantages for detection of any of a number of nucleic acid sequences of interest in the genomic DNA of an individual or organism and has the advantages of being both rapid and extremely accurate in effecting the detection of such mutations. The invention will find wide applicability in diagnosis of a number of genetically associated disorders as well as in other applications where identification of genetic mutations may be important. Exemplary diseases include without limitation, diseases such as cystic fibrosis, generalized myotonia and myotonia congenita, hyperkalemic periodic paralysis, hereditary ovalocytosis, hereditary spherocytosis and glucose malabsorption; which are associated with mutations in the genes encoding ion transporters; multiple endocrine neoplasia, which is associated with mutations in the MEN2a, b, and MEN1 genes; familial medullary thyroid carcinoma, and Hirschsprung's disease, which are associated with mutations in the ret proto-oncogene; familial hypercholesterolemia, which is associated with mutations in the LDL receptor gene; neurofibromatosis and tuberous sclerosis, which are associated with mutations in the NF1 gene, and NF type 2 gene; breast and ovarian cancer, which are associated with mutations in the BRCA1, BRCA2, BRCA3 genes; familial adenomatous polyposis, which is associated with mutations in the APC gene; severe combined immunodeficiency, which is associated with mutations in the adenosine deaminase gene; xeroderma pigmentosum, which is associated with mutations in the XPAC gene; Cockayne's syndrome, which is associated with mutations in the ERCC6 excision repair gene; fragile X, which is associated with mutations in the fmr1 gene; Duchenne's muscular dystrophy, which is associated with mutations in the Duchenne's muscular dystrophy gene; myotonic dystrophy, which is associated with mutations in the myotonic dystrophy protein kinase gene; bulbar muscular dystrophy, which is associated with mutations in the androgen receptor genes; Huntington's disease, which is associated with mutations in the Huntington's gene; Peutz-jegher's syndrome; Lesch-Nyhan syndrome, which is associated with mutations in the HPRT gene; Tay-Sachs disease, which is associated with mutations in the HEXA gene; congenital adrenal hyperplasia, which is associated with mutations in the steroid 21-hydroxylase gene; primary hypertension, which is associated with mutations in the angiotensin gene; hereditary non-polyposis, which is associated with mutations in the hNMLH1 gene; colorectal carcinoma, which is associated with mutations in the 2 mismatch repair genes; colorectal cancer, which is associated with mutations in the APC gene; forms of Alzheimer's disease which have been associated with the

apolipoprotein E gene, retinoblastoma, which is associated with mutations in the Rb gene; Li-Fraumeni syndrome, which is associated with mutations in the p53 gene; various malignancies and diseases that are associated with translocations: e.g., in the bcr/abl, bcl-2 gene; chromosomes 11 to 14 and chromosomes 15 to 17 transpositions. The references at the end of the specification which are expressly incorporated herein by reference describe genetic mutations associated with certain diseases which may be tested for in accordance with the invention as well as sequences provided in GENBANK, the contents of which are also expressly incorporated herein by reference.

Double Stranded Experiment

For the purposes of illustration, the two complementary strands of a double-stranded DNA segment are referred to as strand "A" and strand "B". Either strand may be designated "A" or "B". The wild-type "B" strand oligo (ras codon 12) having the oligonucleotide sequence 5'-GCCTACGCCACCAGCTCCAACTAC-3' (SEQ ID NO. 3) was coupled to 3.0 micrometers (μm) latex microspheres (manufactured by Interfacial Dynamics, Portland, OR) by carbodiimide coupling. Double stranded competitor was prepared by combining equal amounts of both the "A" and "B" strands of either the wild-type or mutant version of the oligo, mutant "B" strand having the sequence 5'-GCCTACGCCACAAGCTCCAACTAC-3' (SEQ ID NO. 4) (ras codon 12) in 5X SSC buffer. Annealing was accomplished by heating the mixture to 65°C for five minutes, then cooling slowly to room temperature. Competitive hybridization was accomplished by combining approximately 40 picomoles of the bead-attached oligo (wild-type "B" strand) with the indicated amounts of double stranded competitor in 2.3X SSC buffer at approximately 25°C. Finally, 100 picomoles of the fluorescinated oligo (wild-type "A" strand) was added to the reaction mixture. This mixture was incubated for two hours at room temperature, and then diluted with 300 μl of saline pH 7.3, and analyzed on the "FACSCAN" (manufactured by Becton-Dickinson Immunocytometry Systems, San Jose, CA). The results are shown in **Table 20** below and in **Figures 43a** through **43c**.

TABLE 20: Double-Stranded Experimental Results Using Wild-Type "B" Oligonucleotide

Double Stranded Competitor (picomole)	Percent Inhibition (%)		Fold Competition Wild-Type/Mutant
	Wild-Type	Mutant	
10	20	9	2.2
100	35	12	2.9
1000	56	17	3.3

These results clearly show that the DNA containing the single point mutation ("Mutant") was a detectably less effective inhibitor of hybridization between the DNA on the beads and the fluorescent oligonucleotide probe at all concentrations of competitor tested.

Single Stranded Experiment

The wild-type "B" strand oligo (ras codon 12) was coupled to 3.0 μm latex microspheres (manufactured by Interfacial Dynamics) by carbodiimide coupling. Competitive hybridization was accomplished by combining approximately 40 picomoles of the bead-attached oligo with 100 picomoles of the fluorescinated oligo (wild-type "A" strand) in 2.3X SSC buffer. Finally, the indicated amounts of single stranded competitor (either mutant or wild-type) were added to two separate aliquots of the reaction mixture. These aliquots were incubated for two hours at room temperature, and then diluted with 300 μl of saline pH 7.3. and analyzed on the FACSCAN flow cytometer. The result of these experiments are set forth in **Table 21** below and in **Figures 44a** and **44b**.

TABLE 21: Single-Stranded Experimental Results

Single Stranded Competitor (picomole)	Percent Inhibition (%) Wild-Type	Mutant	Fold Competition Wild-Type/Mutant
100 "A" Strand	14	6	2.4
1000 "A" Strand	25	11	2.3

These results clearly show that the DNA containing the single point mutation ("Mutant") was a detectably less effective inhibitor of hybridization between the DNA on the beads and the florescent oligonucleotide probe at all concentrations of competitor tested.

Resequencing analysis of PCR products using multiplexed analysis.

This example demonstrates the ability of flow cytometry to perform resequencing analysis of PCR products. As a model system, PCR products were derived from the DQA1 gene, in the region of the gene which determines the major alleles of DQA1. The DQA1 gene represents the DNA coding sequence for the alpha chain of the DQ molecule. DQ is classified as a class II histocompatibility locus and is expressed in allelic form in all humans. Most individuals are heterozygous for DQA , i.e., they express two different DQA alleles. The determination of DQA alleles is used in identity testing for paternity and forensic purposes.

Seventeen alleles of DQA1 have been defined by DNA sequencing; however, eight major alleles account for the large majority of the population. These alleles are determined by fourteen unique DNA sequences contained within four regions of the DQA1 gene; all four regions are contained within a 227 base pair PCR product derived from human genomic DNA.

Flow cytometry was used to determine the presence or absence of all fourteen DNA sequences in a PCR product simultaneously in a single reaction tube, thereby allowing determination of the DQA alleles expressed in a given sample. The system is based on competitive hybridization between the PCR product and complementary oligonucleotide pairs representing each of the fourteen unique DNA sequences. One strand of each oligonucleotide

pair is coupled to a unique subset of microspheres and the complementary strand is labeled with a green emitting fluorophore. After coupling, the fourteen unique microsphere subsets were pooled to produce the mixed bead set. After addition of the fourteen fluorescent oligonucleotides and the PCR product to the beadset, the mixture is hybridized and then analyzed by flow cytometry. The ability of the PCR product to inhibit the hybridization of the complementary fluorescent oligonucleotides to their respective microsphere subsets is used to determine the DNA sequences, and thus, the allele(s) present in the PCR product.

Microspheres: Carboxylate-modified latex (CML) microspheres of 5.5 micron mean diameter were obtained from Bangs Laboratories, Inc. (Carmel, IN). The microspheres were differentially dyed with varying concentrations of two fluorescent dyes with orange and red emission spectra to produce fourteen unique microsphere subsets.

Oligonucleotides: Fourteen oligonucleotide pairs (complementary strands designated "A" and "B") corresponding to allelic sequences within the DQA1 gene (**Table 22**) were synthesized by Oligos, Etc. (Wilsonville, OR). using standard automated techniques. Each eighteen-base oligonucleotide was substituted at the 5' end with an amino-terminal linker during synthesis.

Oligonucleotide coupling to microspheres: The "B" strand of each oligonucleotide pair was coupled to a unique subset of CML microspheres using carbodiimide chemistry. Briefly, 0.1 mL of a 1 mM solution of oligonucleotide in 0.1 M MES (2-[*N*-morpholino]ethanesulfonic acid), pH 4.5 was added to 1.0 mL of microspheres (1% solids) in 0.1 M MES, pH 4.5. To this mixture, 0.05 mL of a 10 mg/mL solution of EDC (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride) was added and mixed vigorously. The mixture was incubated for 30 minutes at room temperature, followed by another addition of EDC, mixing, and incubation as above. Following the second incubation period, the microspheres were pelleted by centrifugation and resuspended in 0.4 mL of 0.1 M MES, pH 4.5 and stored at 4°C.

Oligonucleotide labeling: The "A" strand of each oligonucleotide pair was fluorescently labeled with Bodipy FL-X (6-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)

amino)hexanoic acid, succinimidyl ester) (Molecular Probes, Inc. , Eugene, OR). Briefly, a 400 μL solution containing 20 μM oligonucleotide in 0.1 M sodium bicarbonate and 5% DMSO, pH 8.2 was reacted with 30 μL Bodipy FL-X (10 mg/mL in DMSO) for 16-18 hours at room temperature. The mixture was desalted on a PD10 column equilibrated in TE (10 mM TrisHCl, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0) to remove excess unreacted dye and stored at 4°C.

DNA extraction: Tissue sample (template) DNA was purified using the QIAmp Blood Kit (Qiagen, Chatsworth, CA) for DNA purification. Briefly, 1 x 10^7 tissue culture cells or 200 μL whole blood is lysed with Qiagen protease and Buffer AL. The lysate is incubated at 70°C for 10 minutes followed by addition of 210 μL ethanol. The mixture is applied to a QIAmp spin column and centrifuged at 8,000 x g for 1 minute. The filtrate is discarded, 500 μL Buffer AW is added to the column and the centrifugation is repeated; this step is repeated. The filtrates are discarded and the DNA is eluted into a new tube by addition of 200 μL Buffer AE, incubation at room temperature for 1 minute, followed by centrifugation as above.

Polymerase chain reaction (PCR: PCR primers designated DQA AMP-A (5'-ATGGTGTAAA CTTGTACCAGT-3', SEQ ID NO. 5) and DQA AMP-B (5'-TTGGTAGCAG CGGTAGAGTTG-3', SEQ ID NO. 6) (World Health Organization, 1994) were synthesized by Oligos, Etc. (Wilsonville, OR) using standard automated techniques. PCR was performed with reagents (PCR buffer, dNTPs, $MgCl_2$, and TAQ DNA polymerase) from Life Technologies, Inc.(Gaithersburg, MD). The reaction mixture (50 μL) contained 1 μM of each primer, 200 nM dNTPs, 3 mM $MgCl_2$, 4 - 10 μg/mL DNA template, and 2.5 units TAQ DNA polymerase in PCR buffer. The PCR reaction was performed on an Idaho Technologies thermal cycler (Idaho Falls, ID) using and initial step at 94°C for 45 sec, and 32 cycles of 94°C for 30 sec, 48°C for 60 sec, and 72°C for 60 sec followed by a final hold at 72°C for 7 minutes. Production of the product was verified by agarose electrophoresis and was quantified by size exclusion chromatography on a Superdex 75 (10/30) column (Pharmacia, Piscataway, NJ). The PCR product was used without purification.

Competitive hybridization analysis: The hybridization reaction was performed in a total volume of 40 μL, containing approximately 8,000 of each bead subset for a total of approximately 110,000 microspheres, 50 nM of each fluorescent oligonucleotide, and 10 - 200 nM PCR product, as competitor, in hybridization buffer (3 M trimethyl ammonium chloride, 0.15% sodium dodecyl sulfate, 3 mM EDTA, and 75 mM TrisHCl, pH 8.0). Briefly, the beadset mixture, in hybridization buffer, was equilibrated at 55°C. The mixture of fluorescent oligonucleotides and PCR product was denatured in a boiling water bath for 10 minutes followed by quick-chilling on ice for 2 minutes. The microspheres were added, mixed well, and the entire reaction was allowed to hybridize for 30 minutes at 55°C. Following hybridization, the mixture was diluted to 250 μL using hybridization buffer and analyzed by flow cytometry.

Results

Microspheres for multiple analytes: **Figure 45** illustrates the classification, using orange and red fluorescence, of the fourteen microsphere subsets used in the DQA1 analysis. Each distinct microsphere subset bears one of the fourteen unique oligonucleotide capture probes on its surface. The level of green fluorescence associated with each subset, after hybridization with the fluorescent oligonucleotide probes, is also determined simultaneously, and measures the reactivity of the fluorescent oligonucleotides (and therefore, the reactivity of the PCR product) with each unique oligonucleotide sequence.

Titration of fluorescent oligonucleotide: To optimize the system for detection of PCR products, fluorescent oligonucleotide was titered in the presence or absence of PCR competitor. **Figure 46** illustrates the hybridization of increasing concentrations of fluorescent oligonucleotide "5503A" to microspheres coupled to oligonucleotide "5503B" in the presence or absence of a 200 nM concentration of double-stranded 0301 PCR product which contains the 5503 sequence. In the absence of competitor, the level of "5503A" which hybridizes to the microspheres, detected as FL1, increases in a linear manner and reaches saturation at approximately 10 nM. In the presence of competitor, the binding curve is shifted to the right indicating inhibition of "5503A" hybridization.

Concentration dependence of inhibition and detection of point mutations: **Figure 47** illustrates the inhibition of fluorescent oligonucleotide hybridization by varying concentrations of complementary and point mutant competitors in the presence of a fixed concentration of fluorescent oligonucleotide. The solid lines show the inhibition of hybridization to bead "3401B" induced by competitors 3401 (u) or 3402 (n). The dashed lines show inhibition of hybridization to bead "3402B" induced by competitors 3401(s) or 3402 (l). Even at the lowest competitor concentration (10 nM), there is approximately a two-fold difference between the reactivity of the identical sequence versus the point mutant.

Specificity of the multiple analyte assay: The specificity of the reaction of each DNA competitor sequence with the multiplexed microsphere subsets is illustrated in **Table 23** and **Figure 48**, using double-stranded oligonucleotide competitors. The pattern of reactivity is consistent with the homology of the different oligonucleotides with identical sequences showing maximal reactivity, closely related sequences showing less reactivity, and unrelated sequences showing little or no reactivity.

Allele-specific reactivity patterns: In order to establish the reactivity patterns of the DQA1 alleles in a model system, simulated alleles were prepared by mixing the oligonucleotides representing the DNA sequences that would be present within a single PCR product for a given allele. **Figure 49** illustrates the typing of four simulated alleles of DQA1. By comparison to the allele reactivity chart shown in **Table 24**, it can be seen that each of the simulated alleles types correctly.

Typing of homozygous genomic DNA: To verify the ability of flow cytometry to correctly type PCR products prepared from genomic DNA, samples of DNA of known, homozygous DQA1 type were obtained from the UCLA Tissue Typing Laboratory, Los Angeles, CA. After PCR amplification, these samples were typed using flow cytometry; the results are shown in **Figure 50**. By comparison to the allele reactivity chart **(Table 24)**, it can be seen that the system correctly types these samples.

Typing of heterozygous genomic DNA: To determine the ability of multiplexed flow analysis to accurately type heterozygous DQA1 haplotypes, twenty-five samples of known heterozygous DQA1 type were obtained from the UCLA Tissue Typing Laboratory, Los Angeles, CA. The samples of homozygous DNA used above were added to the panel and all of the samples were coded and typed in a blinded study. The data from this study are presented in **Table 25**. The last column of **Table 25** entitled "Type" indicates whether the haplotype indicated by UCLA and the Luminex analysis agreed. In 34 of 35 samples, the haplotypes reported by both laboratories agreed; sample number 19 was not typed by the UCLA laboratory, but typed clearly as an 0501/0201 heterozygote in the Luminex analysis. Thus, the multiplexed analysis is capable of typing the DQA1 haplotypes with at least 97% accuracy.

These studies have demonstrated that flow cytometry can rapidly and accurately perform resequencing analysis of PCR products. The model system used here required the analysis of fourteen DNA sequences to determine eight different DQA1 alleles. Flow cytometry was able to perform this analysis in a true simultaneous format, using a single sample of a single PCR product in a single reaction tube. The entire analysis, including setup, hybridization, flow analysis, and data collection and analysis can be accomplished within an hour after PCR amplification of the DNA sample. Thus, it is possible to perform tissue typing or other genetic analysis in less than three hours after obtaining a sample of blood, tissue, etcetera, including the time required for extraction of DNA and PCR amplification.

TABLE 22: DQA1 DNA Sequences

Name	Sequence "A" Strand	Sequence "B" Strand	Allele Specificities
DQA2501	TGGCCAGTACACCCATGA (SEQ ID NO. 7)	TCATGGGTGTACTGGCCA (SEQ ID NO. 8)	**0101, 0401, 0501**
DQA2502	TGGCCAGTTCACCCATGA (SEQ ID NO. 9)	TCATGGGTGAACTGGCCA (SEQ ID NO. 10)	**0103, 0201, 0601**
DQA2503	TGGGCAGTACAGCCATGA (SEQ ID NO. 11)	TCATGGCTGTACTGCCCA (SEQ ID NO. 12)	**0301**
DQA3401	GAGATGAGGAGTTCTACG (SEQ ID NO. 13)	CGTAGAACTCCTCATCTC (SEQ ID NO. 14)	**0101, 0104**
DQA3402	GAGATGAGCAGTTCTACG (SEQ ID NO. 15)	CGTAGAACTGCTCATCTC (SEQ ID NO. 16)	**0102, 0103, 0501**
DQA3403	GAGACGAGCAGTTCTACG (SEQ ID NO. 17)	CGTAGAACTGCTCGTCTC (SEQ ID NO. 18)	**0401, 0601**
DQA3404	GAGACGAGGAGTTCTATG (SEQ ID NO. 19)	CATAGAACTCCTCGTCTC (SEQ ID NO. 20)	**0201, 0301**
DQA4101N	ACCTGGAGAGGAAGGAGA (SEQ ID NO. 21)	TCTCCTTCCTCTCCAGGT (SEQ ID NO. 22)	**0101, 0102, 0201, 0301**
DQA4102	ACCTGGAGAAGAAGGAGA (SEQ ID NO. 23)	TCTCCTTCTTCTCCAGGT (SEQ ID NO. 24)	**0103**
DQA4103	ACCTGGGGAGGAAGGAGA (SEQ ID NO. 25)	TCTCCTTCCTCCCCAGGT (SEQ ID NO. 26)	**0401, 0501, 0601**
DQA5501N	TCAGCAAATTTGGAGGTT (SEQ ID NO. 27)	AACCTCCAAATTTGCTGA (SEQ ID NO. 28)	**0101, 0102, 0103**
DQA5502N	TCCACAGACTTAGATTTG (SEQ ID NO. 29)	CAAATCTAAGTCTGTGGA (SEQ ID NO. 30)	**0201**
DQA5503	TCCGCAGATTTAGAAGAT (SEQ ID NO. 31)	ATCTTCTAAATCTGCGGA (SEQ ID NO. 32)	**0301**
DQA5504	TCAGACAATTTAGATTTG (SEQ ID NO. 33)	CAAATCTAAATTGTCTGA (SEQ ID NO. 34)	**0401, 0501, 0601**

Table 23: Specificity of Oligonucleotide Inhibition

	Bead #													
	2501	2502	2503	3401	3402	3403	3404	4101	4102	4103	5501	5502	5503	5504
Oligo														
none	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
2501	**64%**	10%	5%	-1%	-6%	4%	1%	4%	1%	3%	5%	-9%	2%	3%
2502	19%	**77%**	-7%	-2%	0%	1%	-3%	-3%	-1%	-4%	-4%	-4%	-5%	4%
2503	7%	1%	**85%**	1%	2%	4%	-3%	-2%	4%	-1%	1%	-1%	5%	6%
3401	-1%	-1%	-3%	**76%**	1%	6%	1%	3%	2%	2%	5%	-4%	2%	5%
3402	-1%	-8%	-12%	14%	**83%**	11%	-5%	-5%	-3%	-10%	0%	-7%	-4%	1%
3403	-2%	-3%	-1%	0%	7%	**73%**	-1%	2%	1%	-1%	5%	-9%	-4%	2%
3404	5%	5%	4%	6%	2%	8%	**62%**	10%	9%	6%	10%	0%	7%	9%
4101	6%	4%	6%	10%	5%	10%	6%	**79%**	18%	22%	31%	5%	12%	12%
4102	0%	-1%	-4%	0%	-3%	5%	0%	8%	**79%**	3%	7%	-1%	5%	4%
4103	-2%	11%	3%	5%	6%	7%	5%	4%	7%	**71%**	0%	8%	9%	6%
5501	-3%	3%	0%	2%	5%	5%	1%	-1%	6%	1%	**79%**	9%	6%	4%
5502	3%	5%	5%	5%	7%	3%	-1%	1%	2%	-4%	-7%	**86%**	4%	1%
5503	-5%	0%	-6%	1%	2%	0%	-2%	-8%	0%	-7%	-9%	13%	**80%**	-5%
5504	3%	8%	9%	5%	6%	5%	2%	2%	7%	6%	4%	13%	4%	**93%**

TABLE 24. Allele Reactivity Chart

Allele	Pattern	Sequence
0101	(1,1,1,1)	2501 3401 4101 5501
0102	(1,2,1,1)	2501 3402 4101 5501
0103	(2,2,2,1)	2502 3402 4102 5501
0201	(2,4,1,2)	2502 3404 4101 5502
0301	(3,4,1,3)	2503 3404 4101 5503
0401	(1,3,3,4)	2501 3403 4103 5504
0501	(1,2,3,4)	2501 3402 4103 5504
0601	(2,3,3,4)	2502 3403 4103 5504

Table 25. Blinded typing of Genomic DNA Samples for DQA1

	BEAD SUBSET														
	2501	2502	2503	3401	3402	3403	3404	4101	4102	4103	5501	5502	5503	5504	TYPE
1	4%	25%	35%	7%	69%	8%	58%	36%	61%	-1%	78%	2%	75%	-8%	Y
2	13%	-5%	-7%	63%	-4%	-2%	-3%	37%	2%	-9%	77%	0%	1%	-11%	Y
3	19%	-2%	40%	66%	-3%	0%	65%	51%	-2%	-2%	78%	-2%	76%	-14%	Y
4	22%	13%	18%	33%	68%	9%	1%	-3%	6%	33%	-14%	0%	-11%	36%	Y
5	38%	1%	-7%	68%	78%	6%	-1%	40%	-1%	36%	76%	-3%	-5%	48%	Y
6	-13%	-10%	11%	-10%	-10%	-7%	45%	21%	-4%	-12%	-24%	-9%	75%	-19%	Y
7	-7%	8%	-6%	-11%	-8%	19%	27%	7%	-5%	16%	-27%	31%	-6%	30%	Y
8	20%	-5%	-9%	1%	65%	53%	-2%	37%	-2%	30%	76%	-2%	-7%	43%	Y
9	32%	5%	-1%	7%	76%	5%	2%	57%	0%	-1%	84%	-3%	-11%	-25%	Y
10	32%	10%	60%	4%	72%	1%	71%	60%	-4%	7%	83%	-32%	76%	-40%	Y
11	27%	7%	5%	10%	70%	5%	2%	-14%	-5%	45%	-21%	-6%	-18%	44%	Y
12	29%	3%	57%	8%	71%	6%	67%	60%	0%	3%	84%	-4%	77%	-29%	Y
13	25%	8%	2%	66%	-4%	-8%	-8%	47%	-1%	-4%	83%	-12%	-22%	-36%	Y
14	16%	8%	33%	-3%	0%	16%	29%	24%	2%	26%	-25%	-7%	47%	17%	Y
15	7%	19%	8%	-6%	26%	0%	31%	26%	-5%	28%	-31%	36%	-21%	23%	Y
16	32%	9%	2%	18%	76%	8%	5%	-1%	-2%	48%	-4%	-6%	-17%	52%	Y
17	8%	0%	35%	6%	53%	4%	58%	45%	1%	48%	-13%	-1%	77%	41%	Y
18	1%	-2%	55%	3%	1%	1%	75%	54%	4%	6%	-14%	-3%	84%	-12%	Y
19	12%	18%	2%	5%	47%	8%	54%	37%	5%	46%	-11%	46%	1%	39%	?
20	63%	15%	6%	42%	87%	17%	20%	64%	10%	65%	87%	6%	5%	60%	Y
21	44%	62%	8%	23%	76%	13%	73%	70%	11%	20%	88%	64%	4%	1%	Y
22	-6%	26%	5%	4%	-3%	2%	55%	48%	6%	-1%	-7%	58%	1%	-11%	Y
23	56%	67%	13%	38%	87%	22%	24%	12%	75%	63%	89%	15%	15%	63%	Y
24	15%	25%	5%	60%	58%	7%	10%	42%	61%	-4%	90%	-2%	-3%	-17%	Y
25	47%	15%	12%	75%	15%	50%	17%	56%	4%	55%	83%	2%	-5%	51%	Y
26	30%	8%	56%	15%	71%	5%	67%	65%	2%	9%	85%	-1%	82%	-18%	Y
27	13%	10%	10%	7%	27%	21%	5%	1%	3%	47%	-16%	5%	-5%	51%	Y
28	23%	2%	0%	17%	23%	60%	14%	-2%	-1%	58%	-18%	-2%	-3%	59%	Y
29	24%	6%	10%	48%	46%	5%	10%	56%	5%	8%	86%	2%	-2%	-16%	Y
30	38%	12%	11%	73%	14%	48%	7%	55%	6%	55%	84%	1%	-7%	50%	Y
31	-1%	-1%	19%	0%	20%	-1%	26%	29%	-3%	31%	-28%	-4%	70%	31%	Y
32	57%	16%	6%	83%	81%	11%	6%	59%	7%	60%	86%	9%	-1%	53%	Y
33	-13%	17%	24%	2%	-1%	29%	47%	37%	-6%	50%	-11%	0%	80%	52%	Y
34	33%	7%	5%	19%	75%	6%	13%	54%	1%	2%	85%	24%	-3%	-3%	Y
35	-11%	19%	31%	10%	-14%	0%	70%	46%	-4%	-2%	-57%	44%	79%	-8%	Y

Measuring Enzymes with Bead-Based Assays

The invention may also be used in several formats for measurement of enzymes, enzyme inhibitors and other analytes. For example, bead subsets can be generated that are modified with selected fluorescent substrates which can be enzymatically cleaved from the bead, resulting in a loss of fluorescence (F_m). Enzymes that can be detected and measured using the invention include but are not restricted to, protease, glycosidase, nucleotidase, and oxidoreductase. Any enzyme that results in selected bond cleavage can be measured. A cartoon of the action of enzyme on a bead-bound enzyme is shown in **Figure 51a**. An enzyme that acts upon a bead-bound substrate so that the bead-bound substrate becomes fluorescent or loses fluorescence comprises an assay for the level of enzyme affecting such a change. **Figures 51b** and **51c** depict these situations. Alteration of the substrate could be an oxidation or reduction, alteration of a chemical bond such a hydrolysis or other alteration of the bead-bound substrate so that the fluorescence of the substrate is altered in intensity or spectrum.

Enzymes that act upon pro-enzymes (convertases) can be measured using a bead-bound substrate providing the reaction mixture contains the pro-enzyme and beads bearing a substrate that can be acted upon by the active form of the enzyme. (Providing the specificity of each activated enzyme is distinct, a multiplexed assay is achievable in which several pro-enzymes can be measured at the same time.) The sample is introduced into a mixture of pro-enzymes under reaction conditions. After a fixed time interval during which the convertase acts upon the pro-enzyme, the beadsets specific for each enzyme generated from each pro-enzyme are added and the newly generated activities measured in a subsequent time period which is terminated when the beadsets are analyzed by flow cytometry. Such a process for a single pro-enzyme to enzyme conversion is illustrated by the cartoon in **Figure 51d**.

The action of the enzyme can be measured in an indirect but facile manner using a bead bound substrate as depicted in **Figure 51e**. The action of the enzyme on the bead-bound substrate results in the formation or revelation of a ligate for a fluorescent ligand present in the reaction mixture. The bead bearing the modified substrate then becomes fluorescent by virtue of binding of the fluorescent ligand to the newly formed ligate. In practice, the enzyme(s) would

be added to the beadset under reactive conditions. After a defined time period during which the enzyme acts upon the bead bound substrate, the enzyme action would be stopped and the fluorescent ligands added and after a period for association of ligand with the beadsets, the mixture analyzed by flow cytometry. The fluorescent ligands could be of a single reactivity or multiple ligands employed, the critical specificity is that of the enzyme for the substrate.

The bead-bound substrate may be used to detect the activation of enzyme when the enzyme requires a cofactor for activity. Under this circumstance, the level of the cofactor becomes the limiting component of the reaction mixture and determination of the level of cofactor can be measured. Such a configuration is illustrated in **Figure 51f**. The reaction mixture contains the bead-bound substrate as well as the apo-enzyme. After introduction of the analyte (enzyme cofactor), the reaction mixture is held under reactive conditions for a fixed period of time followed by analysis of the beads by flow cytometry, the level of cofactor limits the level of enzyme activity. Providing the enzymes present require different cofactors and have action on different substrate-bearing beadsets, several cofactors could be measured in a single assay mixture.

In short, bead-borne substrates can be used as reagent as are soluble substrates for enzymes. However, because each type of bead bearing a unique substrate can be distinguished, a mixture of bead subsets can be used to measure several enzyme activities simultaneously in the same reaction mixture.

Fluids that can be analyzed using these techniques include plasma, serum, tears, mucus, saliva, urine, pleural fluid, spinal fluid and gastric fluid, sweat, semen, vaginal secretions, fluid from ulcers and other surface eruptions, blisters, and abscesses, and extracts of tissues including biopsies of normal, malignant, and suspect tissues. An assay according to this aspect of the invention proceeds as follows:

1. Beads containing reactive surface groups (one of the following: amino, aldehyde, acid chloride, amidine, phenolic hydroxyl, phenyl amine, carboxyl) are obtained that can be

discriminated on the basis of, for example, forward angle light scatter, C_1, right angle light scatter, C_2, and one of several wavelengths of fluorescence $C_3 \ldots C_n$ which are designated as orange and red fluorescence, for example, and comprise a number of subsets.

2. Subsets thus obtained are derivatized with a peptide (substrate) having a terminal fluorescent green group, for example fluorescein (F_m).
3. Unreacted surface groups and hydrophobic surface of the bead are blocked and the subsets are processed by a particle analyzer and sorter (FACSCAN) and a uniform population of particles are separated which have a low coefficient of variance for F_m. (e.g., 3%).
4. A fluid to be tested is diluted with an appropriate buffer and added to the beadset mixture to allow enzymes present in the sample to react with (cleave) their corresponding substrate on the surfaces of the beads.
5. After a defined period of time, the reaction is stopped and the entire mixture processed by a flow cytometer and results are determined.

The presence of an enzyme in the clinical sample is indicated by loss of fluorescence resulting from the cleavage of the fluorescent F_m substrate from the bead surface. Because the beads are analyzed in a very small volume (e.g., about 6 picoliters) as they are passed through the flow cytometer's laser beam, interference from free fluorescent molecules (cleaved substrate) will not significantly interfere with the assay. This obviates the requirement of washing of the beads prior to assay and simplifies the procedure significantly.

Time

Time measurement is an important feature of the analysis. The essence of the measurement of an enzyme activity is a change in substrate with time. The activity can be determined by setting a period of time during which the clinical sample is in contact with the beads using standard conditions of pH, ionic composition and temperature. Two alternative processes are available for determination of the bead-bound substrate with time, that is the time expired while the enzyme(s) is (are) acting on each beadset(s).

External Time

In this configuration, as each bead is measured by the flow cytometer, the time at which each measurement was obtained is recorded along with the bead's other measurements. Prior to the beginning of the assay, the baseline measurement is determined. Once the enzyme (clinical sample) is added to the bead mixture, the sample analysis begins. As the beads proceed through the instrument, the time data collected is used to determine the length of time that the bead has been exposed to the clinical sample. The F_m data collected over the period of the assay is used to determine the rate of change of substrate on the beads (kinetics) and thus the rate readily derived for each bead subset in the mixture exposed to the clinical sample.

Internal Time

Time can be determined and at the same time a quality control internally generated by including a "timer" bead subset that bears a substrate which is acted on by an enzyme that does not naturally occur in the clinical sample to be tested. The use of non-pathogenic microbial enzymes and substrates with human samples, for example, would suffice. The corresponding "timer" enzyme is added to the dilution buffer so that a known concentration of the "timer" enzyme is present in the buffer. The degree of action of the "timer" enzyme upon the beads in the "timer" subset can be measured as a function of the loss of fluorescence of the beads in the subset to ensure that proper reaction conditions are achieved. The level of fluorescence of the timer beads can thus be used as an internal standard and an estimation of time.

Determination of Enzyme Inhibitors or Regulators

In addition to direct assay of enzymes, an assay of this type may also be used to detect enzyme inhibitors or regulators. In accordance with this variation, samples being tested for inhibitors are added to the beadset followed by the corresponding enzymes. If inhibitors are present, the measured fluorescent (F_m) values will not be decreased to the same extent as a control containing no inhibitors. In accordance with **Figure 52**, in a similar manner, inhibitors of enzyme activators or binders of cofactors can be measured.

The present invention provides numerous advantages and overcomes many problems associated with prior art techniques of multiplexed diagnostic and genetic analysis apparatus and methods. It will be appreciated by those of ordinary skill having the benefit of this disclosure that numerous variations from the foregoing illustration will be possible without departing from the inventive concept described herein. Accordingly, it is the claims set forth below, and not merely the foregoing illustration, which are intended to define the exclusive rights claimed in this application program.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Luminex Corporation, Chandler, Van S.
Fulton, R. Jerrold
Chandler, Mark B.

(ii) TITLE OF INVENTION: Multiplexed Analysis of Clinical Specimens Aparatus and Method

(iii) NUMBER OF SEQUENCES: 34

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Melinda L. Patterson, Arnold, White & Durkee
(B) STREET: P.O. Box 4433
(C) CITY: Houston
(D) STATE: Texas
(E) COUNTRY: USA
(F) ZIP: 77210-4433

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Patterson, Melinda L.

(B) REGISTRATION NUMBER: 33,062

(C) REFERENCE/DOCKET NUMBER: ILAB:005

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (713) 787-1592

(B) TELEFAX: (713) 789-2689

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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His Tyr Gly Ser Leu Pro Gln Lys
1               5
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(2) INFORMATION FOR SEQ ID NO:2:

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(A) LENGTH: 6 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: not relevant

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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Tyr Gly Ser Leu Pro Gln
1               5
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(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCCTACGCCA CCAGCTCCAA CTAC 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCTACGCCA CAAGCTCCAA CTAC 24

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGGTGTAAA CTTGTACCAG T 21

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTGGTAGCAG CGGTAGAGTT G 21

(2) INFORMATION FOR SEQ ID NO:7:

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(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGCCAGTAC ACCCATGA 18

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCATGGGTGT ACTGGCCA 18

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGGCCAGTTC ACCCATGA 18

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(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCATGGGTGA ACTGGCCA 18

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGGGCAGTAC AGCCATGA 18

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCATGGCTGT ACTGCCCA 18

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGATGAGGA GTTCTACG 18

(2) INFORMATION FOR SEQ ID NO:14:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGTAGAACTC CTCATCTC 18

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAGATGAGCA GTTCTACG 18

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGTAGAACTG CTCATCTC 18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAGACGAGCA GTTCTACG 18

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(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

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(C) STRANDEDNESS: single
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

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CATAGAACTC CTCGTCTC 18

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ACCTGGAGAG GAAGGAGA 18

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TCTCCTTCCT CTCCAGGT 18

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

ACCTGGAGAA GAAGGAGA 18

(2) INFORMATION FOR SEQ ID NO:24:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

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TCTCCTTCTT CTCCAGGT 18

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ACCTGGGGAG GAAGGAGA 18

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TCTCCTTCCT CCCCAGGT 18

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCAGCAAATT TGGAGGTT 18

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AACCTCCAAA TTTGCTGA 18

(2) INFORMATION FOR SEQ ID NO:29:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CAAATCTAAG TCTGTGGA 18

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(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TCCGCAGATT TAGAAGAT 18

(2) INFORMATION FOR SEQ ID NO:32:

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(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

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(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TCAGACAATT TAGATTTG 18

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(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

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(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

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MICROFICHE APPENDIX A

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FORM1.FRM - 2

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FORM1.FRM - 3

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      TabIndex        =   31
      Top             =   120
      Width           =   495
   End
   Begin Data Data2
      Caption         =   "Data2"
      Connect         =   ""
      DatabaseName    =   "C:\ACCESS\orbit.MDB"
      Exclusive       =   0   'False
      Height          =   270
      Left            =   8160
      Options         =   0
      ReadOnly        =   0   'False
```

FORM1.FRM - 4

```
      RecordSource     =   "assay"
      Top              =   0
      Visible          =   0   'False
      Width            =   1455
   End
   Begin ComboBox CubeSel
      Height           =   300
      Left             =   120
      Style            =   2  'Dropdown List
      TabIndex         =   30

      Top              =   960
      Width            =   1815
   End
   Begin Data Data1
      Caption          =   "Data1"
      Connect          =   ""
      DatabaseName     =   "C:\ACCESS\orbit.MDB"
      Exclusive        =   0   'False
      Height           =   270
      Left             =   8355
      Options          =   0
      ReadOnly         =   0   'False
      RecordSource     =   "cubes"
      Top              =   0
      Visible          =   0   'False
      Width            =   1140
   End
   Begin SSCommand ResPrint
      Caption          =   "Print"
      Font3D           =   0  'None
      Height           =   495
      Left             =   7920
      Picture          =   (none)
      TabIndex         =   22
      Top              =   6120
      Width            =   1455
   End
   Begin SSCommand ResSave
      Caption          =   "Save"
      Font3D           =   0  'None
      Height           =   495
      Left             =   7920
      Picture          =   (none)
      TabIndex         =   21
      Top              =   5520
      Width            =   1455
   End
   Begin SSCommand ResClear
      Caption          =   "Clear"
      Font3D           =   0  'None
      Height           =   495
      Left             =   7920
      Picture          =   (none)
      TabIndex         =   20
      Top              =   4920
      Width            =   1455
   End
   Begin Grid Grid1
      FixedCols        =   0
      Height           =   2295
```

```
FORM1.FRM - 5

      Left              =   2640
      Rows              =   64
      ScrollBars        =   2  'Vertical
      TabIndex          =   19
      Top               =   4440
      Width             =   5175
   End
   Begin GRAPH Graph1




      AsciiSymbol       =   "0"



      GraphCaption      =   "Classification"

      GraphTitle        =   "Classification"
      GraphType         =   9  'Scatter
      GridStyle         =   3  'Horizontal and Vertical
      Height            =   2295

      Left              =   120

      NumPoints         =   20
      NumSets           =   16


      TabIndex          =   18
      Top               =   4440
      Width             =   2415

   End
   Begin SSPanel Panel3D2
      Alignment         =   0  'Left Justify - TOP
      BackColor         =   &H00C0C0C0&
      BevelInner        =   1  'Inset
      Caption           =   "Operator Insturctions"
      Font3D            =   0  'None
      Height            =   615
      Left              =   120
      TabIndex          =   15
      Top               =   3720
      Width             =   9375
      Begin TextBox OpInst
         Height         =   375
         Left           =   2040
         MultiLine      =   -1  'True
         TabIndex       =   16
         Top            =   120
         Width          =   5655
      End
```

```
FORM1.FRM - 6

      Begin SSCommand OpInstOK
         Caption          =   "OK"
         Font3D           =   0  'None
         Height           =   375
         Left             =   8040
         Picture          =   (none)
         TabIndex         =   17
         Top              =   120
         Width            =   855
      End
   End
   Begin TextBox SIBox
      Height           =   1815
      Left             =   120
      MultiLine        =   -1  'True
      TabIndex         =   14
      Top              =   1680
      Width            =   3735
   End
   Begin SSPanel Panel3D1
      Alignment        =   0  'Left Justify - TOP
      BackColor        =   &H00C0C0C0&
      Caption          =   "Cytometer Status"
      Font3D           =   0  'None
      Height           =   2535
      Left             =   7680
      TabIndex         =   12
      Top              =   960
      Width            =   1815
      Begin PictureBox FCPic
         BackColor        =   &H00C0C0C0&
         BorderStyle      =   0  'None
         Height           =   495
         Index            =   1
         Left             =   1320
         Picture          =   (Icon)
         ScaleHeight      =   492
         ScaleWidth       =   372
         TabIndex         =   25
         Top              =   240
         Visible          =   0   'False
         Width            =   375
      End
      Begin PictureBox FCPic
         BackColor        =   &H00C0C0C0&
         BorderStyle      =   0  'None
         Height           =   495
         Index            =   0
         Left             =   1320
         Picture          =   (Icon)
         ScaleHeight      =   492
         ScaleWidth       =   372
         TabIndex         =   24
         Top              =   240
         Visible          =   0   'False
         Width            =   375
      End
      Begin Label Label10
         Alignment        =   1  'Right Justify
         BackStyle        =   0  'Transparent
         Caption          =   "Pressure"
```

FORM1.FRM - 7

```
      Height          =   255
      Left            =   120
      TabIndex        =   29
      Top             =   2160
      Width           =   1095
   End
   Begin Label Label9
      Alignment       =   1  'Right Justify
      BackStyle       =   0  'Transparent
      Caption         =   "Waste Water"
      Height          =   255
      Left            =   0
      TabIndex        =   28
      Top             =   1560
      Width           =   1215
   End
   Begin Label Label8
      Alignment       =   1  'Right Justify
      BackStyle       =   0  'Transparent
      Caption         =   "Sheath Fluid"
      Height          =   255
      Left            =   120
      TabIndex        =   27
      Top             =   960
      Width           =   1095
   End
   Begin Label Label7
      Alignment       =   1  'Right Justify
      BackStyle       =   0  'Transparent
      Caption         =   "Flow Control"
      Height          =   255
      Left            =   120
      TabIndex        =   26
      Top             =   360
      Width           =   1095
   End
End
Begin SSFrame TstCtl
   Caption         =   "Test Control"
   Font3D          =   0  'None
   Height          =   2535
   Left            =   3960
   TabIndex        =   4
   Top             =   960
   Width           =   1935
   Begin SSCommand TCHalt
      Caption         =   "Counts"
      Enabled         =   0   'False
      Font3D          =   0  'None
      Height          =   495
      Left            =   240
      Picture         =   (none)
      TabIndex        =   7
      Top             =   1680
      Width           =   1455
   End
   Begin SSCommand TCStart
      Caption         =   "Start Test"
      Enabled         =   0   'False
      Font3D          =   0  'None
      Height          =   495
```

```
FORM1.FRM - 8

         Left              =   240
         Picture           =   (none)
         TabIndex          =   6
         Top               =   1080
         Width             =   1455
      End
      Begin SSCommand TCInit
         Caption           =   "Initialize"
         Enabled           =   0   'False
         Font3D            =   0   'None
         Height            =   495
         Left              =   240
         Picture           =   (none)
         TabIndex          =   5
         Top               =   480
         Width             =   1455
      End
   End
   Begin SSFrame MacCtl
      Caption           =   "Machine Control"
      Font3D            =   0   'None
      Height            =   2535
      Left              =   5880
      TabIndex          =   3
      Top               =   960
      Width             =   1815
      Begin SSCommand MCEnd
         Caption           =   "Exit"
         Font3D            =   0   'None
         Height            =   495
         Left              =   240
         Picture           =   (none)
         TabIndex          =   10
         Top               =   1680
         Width             =   1335
      End
      Begin SSCommand MCAdjust
         Caption           =   "Manual Adjust"
         Font3D            =   0   'None
         Height            =   495
         Left              =   240
         Picture           =   (none)
         TabIndex          =   9
         Top               =   1080
         Width             =   1335
      End
      Begin SSCommand MCCalib
         Caption           =   "Calibrate"
         Font3D            =   0   'None
         Height            =   495
         Left              =   240
         Picture           =   (none)
         TabIndex          =   8
         Top               =   480
         Width             =   1335
      End
   End
   Begin ComboBox AssaySel
      Height            =   300
      Left              =   2040
      Style             =   2   'Dropdown List
```

```
FORM1.FRM - 9

      TabIndex          =   2

      Top               =   960
      Width             =   1815
   End
   Begin Label Label6
      Alignment         =   2  'Center
      BackStyle         =   0  'Transparent
      BorderStyle       =   1  'Fixed Single
      Caption           =   "Results"
      FontBold          =   -1  'True
      FontItalic        =   -1  'True
      FontName          =   "MS Sans Serif"
      FontSize          =   12
      FontStrikethru    =   0   'False
      FontUnderline     =   0   'False
      ForeColor         =   &H00000080&
      Height            =   375
      Left              =   7920
      TabIndex          =   23
      Top               =   4440
      Width             =   1455
   End
   Begin Label Label4
      BackStyle         =   0  'Transparent
      Caption           =   "Sample Identification"
      Height            =   255
      Left              =   120
      TabIndex          =   13
      Top               =   1440
      Width             =   2535
   End
   Begin Line Line1
      X1                =   0
      X2                =   9480
      Y1                =   3600
      Y2                =   3600
   End
   Begin Label Label2
      BackStyle         =   0  'Transparent
      Caption           =   "Specific Assay"
      Height            =   255
      Left              =   2040
      TabIndex          =   0
      Top               =   720
      Width             =   1575
   End
   Begin Label Label1
      BackStyle         =   0  'Transparent
      Caption           =   "Assay Cube"
      Height            =   255
      Left              =   120
      TabIndex          =   1
      Top               =   720
      Width             =   1575
   End
   Begin Label Label3
      Alignment         =   2  'Center
      BackStyle         =   0  'Transparent
      Caption           =   "Orbit Diagnostic Operating System"
      FontBold          =   -1  'True
```

```
FORM1.FRM - 10

      FontItalic        =   0   'False
      FontName          =   "MS Sans Serif"
      FontSize          =   18
      FontStrikethru    =   0   'False
      FontUnderline     =   0   'False
      ForeColor         =   &H00808000&
      Height            =   495
      Left              =   120
      TabIndex          =   11
      Top               =   120
      Width             =   9255
   End
End
```

```
FORM2.FRM - 1

VERSION 2.00
Begin Form Form2
   BorderStyle      =   3  'Fixed Double
   Caption          =   "Manual Adjust"
   ClientHeight     =   6612
   ClientLeft       =   1212
   ClientTop        =   1128
   ClientWidth      =   5508
   Height           =   7032
   Left             =   1164
   LinkTopic        =   "Form2"
   MaxButton        =   0   'False
   MinButton        =   0   'False
   ScaleHeight      =   6612
   ScaleWidth       =   5508
   Top              =   756
   Width            =   5604
   Begin SSFrame Frame3D5
      Caption          =   "DDM Select"
      Font3D           =   3  'Inset w/light shading
      ForeColor        =   &H00000000&
      Height           =   735
      Left             =   2760
      TabIndex         =   63
      Top              =   5880
      Width            =   1455
      Begin OptionButton Option1
         BackColor        =   &H000000FF&
         Caption          =   "Option1"
         Height           =   255
         Index            =   2
         Left             =   1080
         TabIndex         =   66
         Top              =   360
         Width            =   255
      End
      Begin OptionButton Option1
         BackColor        =   &H000080FF&
         Caption          =   "Option1"
         Height           =   255
         Index            =   1
         Left             =   600
         TabIndex         =   65
         Top              =   360
         Width            =   255
      End
      Begin OptionButton Option1
         BackColor        =   &H0000FF00&
         Caption          =   "Option1"
         Height           =   255
         Index            =   0
         Left             =   120
         TabIndex         =   64
         Top              =   360
         Value            =   -1  'True
         Width            =   255
      End
      Begin Shape Shape3
         BackColor        =   &H000000FF&
         BackStyle        =   1  'Opaque
         Height           =   495
```

```
FORM2.FRM - 2

         Left             =   960
         Top              =   240
         Width            =   495
      End
      Begin Shape Shape2
         BackColor        =   &H000080FF&
         BackStyle        =   1  'Opaque
         Height           =   495
         Left             =   480
         Top              =   240
         Width            =   495
      End
      Begin Shape Shape1
         BackColor        =   &H0000FF00&
         BackStyle        =   1  'Opaque
         Height           =   495
         Left             =   0
         Top              =   240
         Width            =   495
      End
   End
   Begin SSPanel Panel3D3
      Alignment        =   0  'Left Justify - TOP
      BackColor        =   &H00C0C0C0&
      BevelInner       =   1  'Inset
      BorderWidth      =   4
      Font3D           =   0  'None
      Height           =   1095
      Left             =   4200
      TabIndex         =   56
      Top              =   2880
      Width            =   1335
      Begin PictureBox Picture1
         BackColor        =   &H00FFFF80&
         BorderStyle      =   0  'None
         Height           =   855
         Left             =   120
         Picture          =   (Icon)
         ScaleHeight      =   852
         ScaleWidth       =   1092
         TabIndex         =   57
         Top              =   120
         Width            =   1095
         Begin Data facset
            Caption          =   "Data1"
            Connect          =   ""
            DatabaseName     =   "C:\ACCESS\orbit.MDB"
            Exclusive        =   0   'False
            Height           =   270
            Left             =   0
            Options          =   0
            ReadOnly         =   0   'False
            RecordSource     =   "facs_settings"
            Top              =   600
            Visible          =   0   'False
            Width            =   1140
         End
         Begin Label Label11
            BackStyle        =   0  'Transparent
            Caption          =   "ORBIT"
            ForeColor        =   &H00808000&
```

FORM2.FRM - 3

```
            Height          =   255
            Left            =   360
            TabIndex        =   58
            Top             =   480
            Width           =   735
         End
      End
   End
   Begin SSPanel Panel3D2
      Alignment       =   0  'Left Justify - TOP
      BackColor       =   &H00C0C0C0&
      BevelInner      =   1  'Inset
      BorderWidth     =   4
      Font3D          =   0  'None
      Height          =   2655
      Left            =   4200
      TabIndex        =   54
      Top             =   3960
      Width           =   1335
      Begin SSCommand Command3D5
         Caption         =   "Done"
         Font3D          =   0  'None
         Height          =   495
         Left            =   120
         Picture         =   (none)
         TabIndex        =   62
         Top             =   1920
         Width           =   1095
      End
      Begin SSCommand Command3D4
         Caption         =   "Save"
         Font3D          =   0  'None
         Height          =   495
         Left            =   120
         Picture         =   (none)
         TabIndex        =   61
         Top             =   1320
         Width           =   1095
      End
      Begin SSCommand Command3D3
         Caption         =   "Reset"
         Font3D          =   0  'None
         Height          =   495
         Left            =   120
         Picture         =   (none)
         TabIndex        =   60
         Top             =   720
         Width           =   1095
      End
      Begin SSCommand Command3D2
         Caption         =   "Set"
         Font3D          =   0  'None
         Height          =   495
         Left            =   120
         Picture         =   (none)
         TabIndex        =   59
         Top             =   120
         Width           =   1095
      End
      Begin SSCommand Command3D1
         Caption         =   "exit"
```

FORM2.FRM - 4

```
      Font3D          =   0  'None
      Height          =   375
      Left            =   240
      Picture         =   (none)
      TabIndex        =   55
      Top             =   2880
      Width           =   735
   End
End
Begin SSPanel Panel3D1
   Alignment       =   0  'Left Justify - TOP
   BackColor       =   &H00C0C0C0&
   BevelInner      =   1  'Inset
   BorderWidth     =   4
   Caption         =   "Status"
   Font3D          =   0  'None
   Height          =   2895
   Left            =   4200
   TabIndex        =   53
   Top             =   0
   Width           =   1335
   Begin Label Stat
      Alignment       =   2  'Center
      BackStyle       =   0  'Transparent
      Caption         =   "Label15"
      Height          =   255
      Index           =   3
      Left            =   120
      TabIndex        =   74
      Top             =   2520
      Width           =   1095
   End
   Begin Label Label15
      Alignment       =   2  'Center
      BackStyle       =   0  'Transparent
      Caption         =   "DDM"
      Height          =   255
      Left            =   120
      TabIndex        =   73
      Top             =   2280
      Width           =   1095
   End
   Begin Label Stat
      Alignment       =   2  'Center
      BackStyle       =   0  'Transparent
      Caption         =   "Label15"
      Height          =   255
      Index           =   2
      Left            =   120
      TabIndex        =   72
      Top             =   1920
      Width           =   1095
   End
   Begin Label Stat
      Alignment       =   2  'Center
      BackStyle       =   0  'Transparent
      Caption         =   "Label15"
      Height          =   255
      Index           =   1
      Left            =   120
      TabIndex        =   71
```

FORM2.FRM - 5

```
      Top              =   1320
      Width            =   1095
   End
   Begin Label Stat
      Alignment        =   2  'Center
      BackStyle        =   0  'Transparent
      Caption          =   "Label15"
      Height           =   255
      Index            =   0
      Left             =   120
      TabIndex         =   70
      Top              =   720
      Width            =   1095
   End
   Begin Label Label14
      Alignment        =   2  'Center
      BackStyle        =   0  'Transparent
      Caption          =   "Samp. Volts"
      Height           =   255
      Left             =   120
      TabIndex         =   69
      Top              =   1680
      Width            =   1095
   End
   Begin Label Label13
      Alignment        =   2  'Center
      BackStyle        =   0  'Transparent
      Caption          =   "Laser Amps"
      Height           =   255
      Left             =   120
      TabIndex         =   68
      Top              =   1080
      Width            =   1095
   End
   Begin Label Label12
      Alignment        =   2  'Center
      BackStyle        =   0  'Transparent
      Caption          =   "Laser Volts"
      Height           =   255
      Left             =   120
      TabIndex         =   67
      Top              =   480
      Width            =   1095
   End
End
Begin SSFrame Frame3D4
   Caption          =   "DDM Amp"
   Font3D           =   3  'Inset w/light shading
   ForeColor        =   &H00000000&
   Height           =   975
   Left             =   0
   TabIndex         =   46
   Top              =   5640
   Width            =   2775
   Begin HScrollBar ddmscroll
      Height           =   255
      Index            =   0
      LargeChange      =   10
      Left             =   720
      Max              =   999
      TabIndex         =   51
```

FORM2.FRM - 6

```
      Top              =   240
      Width            =   1335
   End
   Begin TextBox ddmtxt
      Height           =   285
      Index            =   0
      Left             =   2160
      TabIndex         =   50
      Text             =   "Text1"
      Top              =   240
      Width            =   495
   End
   Begin HScrollBar ddmscroll
      Height           =   255
      Index            =   1
      LargeChange      =   10
      Left             =   720
      Max              =   999
      TabIndex         =   48
      Top              =   600
      Width            =   1335
   End
   Begin TextBox ddmtxt
      Height           =   285
      Index            =   1
      Left             =   2160
      TabIndex         =   47
      Text             =   "Text1"
      Top              =   600
      Width            =   495
   End
   Begin Label Label10
      BackStyle        =   0  'Transparent
      Caption          =   "FLA"
      Height           =   255
      Index            =   5
      Left             =   120
      TabIndex         =   52
      Top              =   240
      Width            =   615
   End
   Begin Label Label10
      BackStyle        =   0  'Transparent
      Caption          =   "FLW"
      Height           =   255
      Index            =   4
      Left             =   120
      TabIndex         =   49
      Top              =   600
      Width            =   615
   End
End
Begin SSFrame Frame3D3
   Caption          =   "Trigger Level"
   Font3D           =   3  'Inset w/light shading
   ForeColor        =   &H00000000&
   Height           =   2055
   Left             =   2760
   TabIndex         =   38
   Top              =   3840
   Width            =   1455
```

FORM2.FRM - 7

```
Begin VScrollBar VScroll1
   Height          =   1575
   LargeChange     =   10
   Left            =   960
   Max             =   999
   TabIndex        =   41
   Top             =   360
   Width           =   255
End
Begin SSOption trigger
   Caption         =   "FL3"
   Font3D          =   0  'None
   Height          =   255
   Index           =   4
   Left            =   120
   TabIndex        =   45
   Top             =   1680
   Width           =   615
End
Begin SSOption trigger
   Caption         =   "FL2"
   Font3D          =   0  'None
   Height          =   255
   Index           =   3
   Left            =   120
   TabIndex        =   44
   Top             =   1440
   Width           =   615
End
Begin SSOption trigger
   Caption         =   "FL1"
   Font3D          =   0  'None
   Height          =   255
   Index           =   2
   Left            =   120
   TabIndex        =   43
   Top             =   1200
   Width           =   615
End
Begin SSOption trigger
   Caption         =   "SSC"
   Font3D          =   0  'None
   Height          =   255
   Index           =   1
   Left            =   120
   TabIndex        =   42
   Top             =   960
   Width           =   615
End
Begin TextBox trigval
   Height          =   285
   Left            =   120
   TabIndex        =   40
   Text            =   "Text2"
   Top             =   360
   Width           =   615
End
Begin SSOption trigger
   Caption         =   "FSC"
   Font3D          =   0  'None
   Height          =   255
```

```
FORM2.FRM - 8

         Index           =   0
         Left            =   120
         TabIndex        =   39
         Top             =   720
         Width           =   615
      End
   End
   Begin SSFrame Frame3D2
      Caption          =   "Compensation"
      Font3D           =   3  'Inset w/light shading
      ForeColor        =   &H00000000&
      Height           =   1815
      Left             =   0
      TabIndex         =   25
      Top              =   3840
      Width            =   2775
      Begin TextBox Text1
         Height           =   285
         Index            =   3
         Left             =   2160
         TabIndex         =   33
         Text             =   "Text1"
         Top              =   1440
         Width            =   495
      End
      Begin TextBox Text1
         Height           =   285
         Index            =   2
         Left             =   2160
         TabIndex         =   32
         Text             =   "Text1"
         Top              =   1080
         Width            =   495
      End
      Begin TextBox Text1
         Height           =   285
         Index            =   1
         Left             =   2160
         TabIndex         =   31
         Text             =   "Text1"
         Top              =   720
         Width            =   495
      End
      Begin TextBox Text1
         Height           =   285
         Index            =   0
         Left             =   2160
         TabIndex         =   30
         Text             =   "Text1"
         Top              =   360
         Width            =   495
      End
      Begin HScrollBar HScroll1
         Height           =   255
         Index            =   3
         LargeChange      =   10
         Left             =   720
         Max              =   999
         TabIndex         =   29
         Top              =   1440
         Width            =   1335
```

FORM2.FRM - 9

```
End
Begin HScrollBar HScroll1
   Height          =   255
   Index           =   2
   LargeChange     =   10
   Left            =   720
   Max             =   999
   TabIndex        =   28
   Top             =   1080
   Width           =   1335
End
Begin HScrollBar HScroll1
   Height          =   255
   Index           =   1
   LargeChange     =   10
   Left            =   720
   Max             =   999
   TabIndex        =   27
   Top             =   720
   Width           =   1335
End
Begin HScrollBar HScroll1
   Height          =   255
   Index           =   0
   LargeChange     =   10
   Left            =   720
   Max             =   999
   TabIndex        =   26
   Top             =   360
   Width           =   1335
End
Begin Label Label10
   BackStyle       =   0  'Transparent
   Caption         =   "FL3-2"
   Height          =   255
   Index           =   3
   Left            =   120
   TabIndex        =   37
   Top             =   1440
   Width           =   615
End
Begin Label Label10
   BackStyle       =   0  'Transparent
   Caption         =   "FL2-3"
   Height          =   255
   Index           =   2
   Left            =   120
   TabIndex        =   36
   Top             =   1080
   Width           =   615
End
Begin Label Label10
   BackStyle       =   0  'Transparent
   Caption         =   "FL2-1"
   Height          =   255
   Index           =   1
   Left            =   120
   TabIndex        =   35
   Top             =   720
   Width           =   615
End
```

```
FORM2.FRM - 10

      Begin Label Label10
         BackStyle       =   0  'Transparent
         Caption         =   "FL1-2"
         Height          =   255
         Index           =   0
         Left            =   120
         TabIndex        =   34
         Top             =   360
         Width           =   615
      End
   End
   Begin SSFrame modefr
      Caption          =   "Mode"
      Font3D           =   3  'Inset w/light shading
      ForeColor        =   &H00000000&
      Height           =   975
      Left             =   0
      TabIndex         =   14
      Top              =   2880
      Width            =   4215
      Begin SSRibbon fl3mod
         AutoSize         =   0  'None
         BackColor        =   &H00C0C0C0&
         GroupAllowAllUp  =   0   'False
         GroupNumber      =   5
         Height           =   240
         Index            =   1
         Left             =   2760
         PictureDisabled  =   (none)
         PictureDn        =   (none)
         PictureDnChange  =   1  'Dither 'PictureUp' Bitmap
         PictureUp        =   (none)
         Top              =   600
         Value            =   -1  'True
         Width            =   495
      End
      Begin SSRibbon fl3mod
         AutoSize         =   0  'None
         BackColor        =   &H00C0C0C0&
         GroupAllowAllUp  =   0   'False
         GroupNumber      =   5
         Height           =   240
         Index            =   0
         Left             =   2760
         PictureDisabled  =   (none)
         PictureDn        =   (none)
         PictureDnChange  =   1  'Dither 'PictureUp' Bitmap
         PictureUp        =   (none)
         Top              =   360
         Width            =   495
      End
      Begin SSRibbon fl2mod
         AutoSize         =   0  'None
         BackColor        =   &H00C0C0C0&
         GroupAllowAllUp  =   0   'False
         GroupNumber      =   4
         Height           =   240
         Index            =   1
         Left             =   2280
         PictureDisabled  =   (none)
         PictureDn        =   (none)
```

FORM2.FRM - 11

```
      PictureDnChange =   1  'Dither 'PictureUp' Bitmap
      PictureUp       =   (none)
      Top             =   600
      Value           =   -1  'True
      Width           =   495
   End
   Begin SSRibbon fl2mod
      AutoSize        =   0  'None
      BackColor       =   &H00C0C0C0&
      GroupAllowAllUp =   0   'False
      GroupNumber     =   4
      Height          =   240
      Index           =   0
      Left            =   2280
      PictureDisabled =   (none)
      PictureDn       =   (none)
      PictureDnChange =   1  'Dither 'PictureUp' Bitmap
      PictureUp       =   (none)
      Top             =   360
      Width           =   495
   End
   Begin SSRibbon fl1mod
      AutoSize        =   0  'None
      BackColor       =   &H00C0C0C0&
      GroupAllowAllUp =   0   'False
      GroupNumber     =   3
      Height          =   240
      Index           =   1
      Left            =   1800
      PictureDisabled =   (none)
      PictureDn       =   (none)
      PictureDnChange =   1  'Dither 'PictureUp' Bitmap
      PictureUp       =   (none)
      Top             =   600
      Value           =   -1  'True
      Width           =   495
   End
   Begin SSRibbon fl1mod
      AutoSize        =   0  'None
      BackColor       =   &H00C0C0C0&
      GroupAllowAllUp =   0   'False
      GroupNumber     =   3
      Height          =   240
      Index           =   0
      Left            =   1800
      PictureDisabled =   (none)
      PictureDn       =   (none)
      PictureDnChange =   1  'Dither 'PictureUp' Bitmap
      PictureUp       =   (none)
      Top             =   360
      Width           =   495
   End
   Begin SSRibbon sscmod
      AutoSize        =   0  'None
      BackColor       =   &H00C0C0C0&
      GroupAllowAllUp =   0   'False
      GroupNumber     =   2
      Height          =   240
      Index           =   1
      Left            =   1320
      PictureDisabled =   (none)
```

FORM2.FRM - 12

```
      PictureDn        =   (none)
      PictureDnChange  =   1  'Dither 'PictureUp' Bitmap
      PictureUp        =   (none)
      Top              =   600
      Value            =   -1  'True
      Width            =   495
   End
   Begin SSRibbon sscmod
      AutoSize         =   0  'None
      BackColor        =   &H00C0C0C0&
      GroupAllowAllUp  =   0   'False
      GroupNumber      =   2
      Height           =   240
      Index            =   0
      Left             =   1320
      PictureDisabled  =   (none)
      PictureDn        =   (none)
      PictureDnChange  =   1  'Dither 'PictureUp' Bitmap
      PictureUp        =   (none)
      Top              =   360
      Width            =   495
   End
   Begin SSRibbon fscmod
      AutoSize         =   0  'None
      BackColor        =   &H00C0C0C0&
      GroupAllowAllUp  =   0   'False
      Height           =   240
      Index            =   1
      Left             =   840
      PictureDisabled  =   (none)
      PictureDn        =   (none)
      PictureDnChange  =   1  'Dither 'PictureUp' Bitmap
      PictureUp        =   (none)
      Top              =   600
      Value            =   -1  'True
      Width            =   495
   End
   Begin SSRibbon fscmod
      AutoSize         =   0  'None
      BackColor        =   &H00C0C0C0&
      GroupAllowAllUp  =   0   'False
      Height           =   240
      Index            =   0
      Left             =   840
      PictureDisabled  =   (none)
      PictureDn        =   (none)
      PictureDnChange  =   1  'Dither 'PictureUp' Bitmap
      PictureUp        =   (none)
      Top              =   360
      Width            =   495
   End
   Begin Label Label9
      Alignment        =   2  'Center
      BackStyle        =   0  'Transparent
      Caption          =   "F13"
      Height           =   255
      Left             =   2760
      TabIndex         =   15
      Top              =   120
      Width            =   495
   End
```

FORM2.FRM - 13

```
Begin Label Label8
   Alignment        =   2  'Center
   BackStyle        =   0  'Transparent
   Caption          =   "FL2"
   ForeColor        =   &H00000000&
   Height           =   255
   Left             =   2280
   TabIndex         =   16
   Top              =   120
   Width            =   495
End
Begin Label Label7
   Alignment        =   2  'Center
   BackStyle        =   0  'Transparent
   Caption          =   "FL1"
   ForeColor        =   &H00000000&
   Height           =   255
   Left             =   1800
   TabIndex         =   17
   Top              =   120
   Width            =   495
End
Begin Label Label6
   Alignment        =   2  'Center
   BackStyle        =   0  'Transparent
   Caption          =   "SSC"
   Height           =   255
   Left             =   1320
   TabIndex         =   20
   Top              =   120
   Width            =   495
End
Begin Label Label4
   Alignment        =   2  'Center
   BackColor        =   &H00000000&
   BackStyle        =   0  'Transparent
   Caption          =   "FSC"
   ForeColor        =   &H00000000&
   Height           =   255
   Left             =   840
   TabIndex         =   21
   Top              =   120
   Width            =   495
End
Begin Label Label5
   BackStyle        =   0  'Transparent
   Caption          =   "Log"
   Height           =   255
   Left             =   120
   TabIndex         =   24
   Top              =   360
   Width            =   615
End
Begin Label Linear
   BackStyle        =   0  'Transparent
   Caption          =   "Linear"
   Height           =   255
   Left             =   120
   TabIndex         =   23
   Top              =   600
   Width            =   855
```

FORM2.FRM - 14

```
      End
   End
   Begin SSFrame Frame3D1
      Caption         =   "Amplifier"
      Font3D          =   3  'Inset w/light shading
      ForeColor       =   &H00000000&
      Height          =   1455
      Left            =   0
      TabIndex        =   8
      Top             =   1440
      Width           =   4215
      Begin SpinButton Spina
         BackColor        =   &H000000FF&
         Delay            =   100
         ForeColor        =   &H00C0C0C0&
         Height           =   495
         Index            =   4
         Left             =   3360
         LightColor       =   &H000000C0&
         ShadeColor       =   &H00000080&
         ShadowBackColor  =   &H000000FF&
         ShadowForeColor  =   &H000000FF&
         SpinBackColor    =   &H000000FF&
         SpinForeColor    =   &H00404040&
         TdThickness      =   2
         Top              =   720
         Width            =   495
      End
      Begin SpinButton Spina
         BackColor        =   &H000080FF&
         Delay            =   100
         ForeColor        =   &H00C0C0C0&
         Height           =   495
         Index            =   3
         Left             =   2640
         LightColor       =   &H000080FF&
         ShadeColor       =   &H00404080&
         ShadowBackColor  =   &H000080FF&
         ShadowForeColor  =   &H000080FF&
         SpinBackColor    =   &H000080FF&
         SpinForeColor    =   &H00404040&
         TdThickness      =   2
         Top              =   720
         Width            =   495
      End
      Begin SpinButton Spina
         BackColor        =   &H0000FF00&
         Delay            =   100
         ForeColor        =   &H00C0C0C0&
         Height           =   495
         Index            =   2
         Left             =   1920
         LightColor       =   &H0000C000&
         ShadeColor       =   &H00008000&
         ShadowBackColor  =   &H0000FF00&
         ShadowForeColor  =   &H0000FF00&
         SpinBackColor    =   &H0000FF00&
         SpinForeColor    =   &H00404040&
         TdThickness      =   2
         Top              =   720
         Width            =   495
```

FORM2.FRM - 15

```
End
Begin SpinButton Spina
   BackColor        =   &H00FFFF00&
   Delay            =   100
   Height           =   495
   Index            =   1
   Left             =   1080
   SpinBackColor    =   &H00FFFF00&
   TdThickness      =   1
   Top              =   720
   Width            =   495
End
Begin SpinButton Spina
   BackColor        =   &H0080FF80&
   ForeColor        =   &H00FFFF00&
   Height           =   495
   Index            =   0
   Left             =   360
   LightColor       =   &H00FFFF80&
   ShadeColor       =   &H00FFFF80&
   ShadowForeColor  =   &H0080FF80&
   SpinBackColor    =   &H0080FF80&
   SpinForeColor    =   &H80000008&
   TdThickness      =   1
   Top              =   720
   Width            =   495
End
Begin TextBox amp
   Height           =   285
   Index            =   4
   Left             =   3360
   TabIndex         =   13
   Text             =   "Text1"
   Top              =   360
   Width            =   495
End
Begin TextBox amp
   Height           =   285
   Index            =   3
   Left             =   2640
   TabIndex         =   12
   Text             =   "Text1"
   Top              =   360
   Width            =   495
End
Begin TextBox amp
   Height           =   285
   Index            =   2
   Left             =   1920
   TabIndex         =   11
   Text             =   "Text1"
   Top              =   360
   Width            =   495
End
Begin TextBox amp
   Height           =   285
   Index            =   1
   Left             =   1080
   TabIndex         =   10
   Text             =   "Text1"
   Top              =   360
```

FORM2.FRM - 16

```
      Width           =   495
   End
   Begin TextBox amp
      Height          =   285
      Index           =   0
      Left            =   360
      TabIndex        =   9
      Text            =   "Text1"
      Top             =   360
      Width           =   495
   End
   Begin Label Label3
      Alignment       =   2  'Center
      BackStyle       =   0  'Transparent
      Caption         =   "SSC"
      Height          =   255
      Left            =   1080
      TabIndex        =   22
      Top             =   1200
      Width           =   495
   End
   Begin Label Label1
      Alignment       =   2  'Center
      BackStyle       =   0  'Transparent
      Caption         =   "FSC"
      Height          =   255
      Index           =   4
      Left            =   360
      TabIndex        =   19
      Top             =   1200
      Width           =   495
   End
   Begin Label Label1
      Alignment       =   2  'Center
      BackStyle       =   0  'Transparent
      Caption         =   "FSC"
      Height          =   15
      Index           =   3
      Left            =   0
      TabIndex        =   18
      Top             =   240
      Width           =   495
   End
End
Begin TextBox txtNumber
   Height          =   285
   Index           =   4
   Left            =   3360
   TabIndex        =   5
   Text            =   "Text1"
   Top             =   360
   Width           =   495
End
Begin TextBox txtNumber
   Height          =   285
   Index           =   3
   Left            =   2640
   TabIndex        =   4
   Text            =   "Text1"
   Top             =   360
   Width           =   495
```

FORM2.FRM - 17

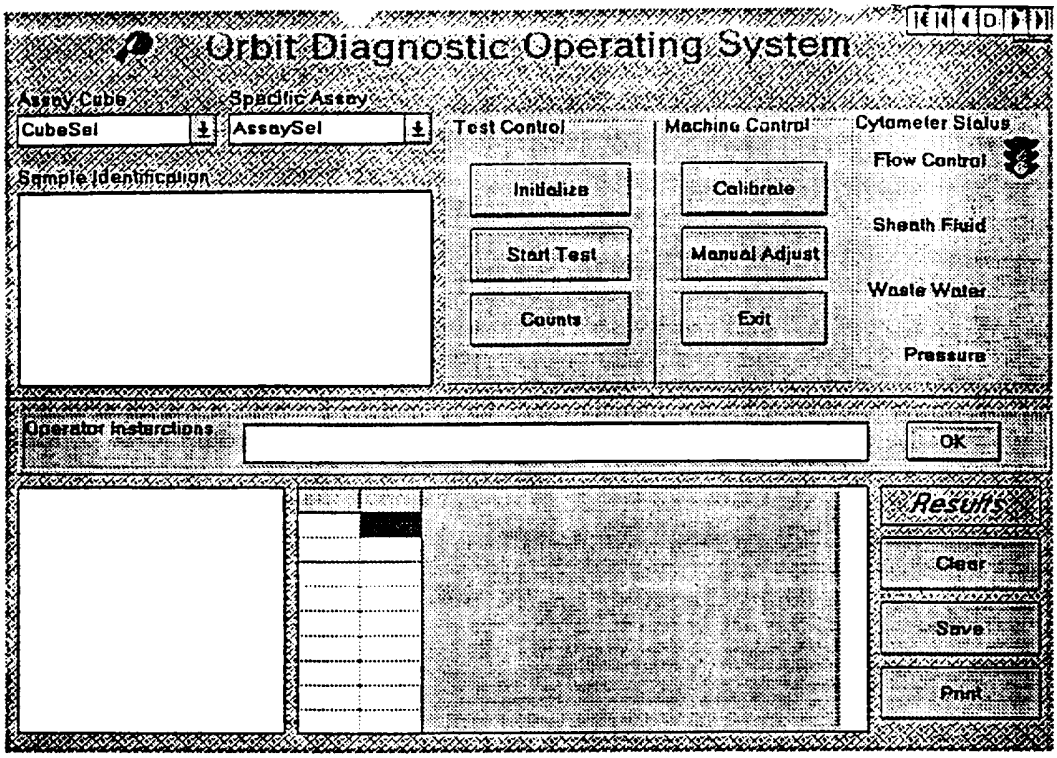
```
   End
   Begin TextBox txtNumber
      Height          =   285
      Index           =   2
      Left            =   1920
      TabIndex        =   3
      Text            =   "Text1"
      Top             =   360
      Width           =   495
   End
   Begin TextBox txtNumber
      Height          =   285
      Index           =   1
      Left            =   1080
      TabIndex        =   2
      Text            =   "Text1"
      Top             =   360
      Width           =   495
   End
   Begin SpinButton Spin1
      BackColor       =   &H0000FF00&
      Delay           =   100
      ForeColor       =   &H00C0C0C0&
      Height          =   495
      Index           =   2
      Left            =   1920
      LightColor      =   &H0000C000&
      ShadeColor      =   &H00008000&
      ShadowBackColor =   &H0000FF00&
      ShadowForeColor =   &H0000FF00&
      SpinBackColor   =   &H0000FF00&
      SpinForeColor   =   &H00404040&
      TdThickness     =   2
      Top             =   720
      Width           =   495
   End
   Begin SpinButton Spin1
      BackColor       =   &H00FFFF00&
      Delay           =   100
      Height          =   495
      Index           =   1
      Left            =   1080
      SpinBackColor   =   &H00FFFF00&
      TdThickness     =   1
      Top             =   720
      Width           =   495
   End
   Begin SSFrame PMT
      Caption         =   "Photo Multiplier"
      Font3D          =   3  'Inset w/light shading
      Height          =   1455
      Index           =   0
      Left            =   0
      TabIndex        =   0
      Top             =   0
      Width           =   4215
      Begin SpinButton Spin1
         BackColor       =   &H000000FF&
         Delay           =   100
         ForeColor       =   &H00C0C0C0&
         Height          =   495
```

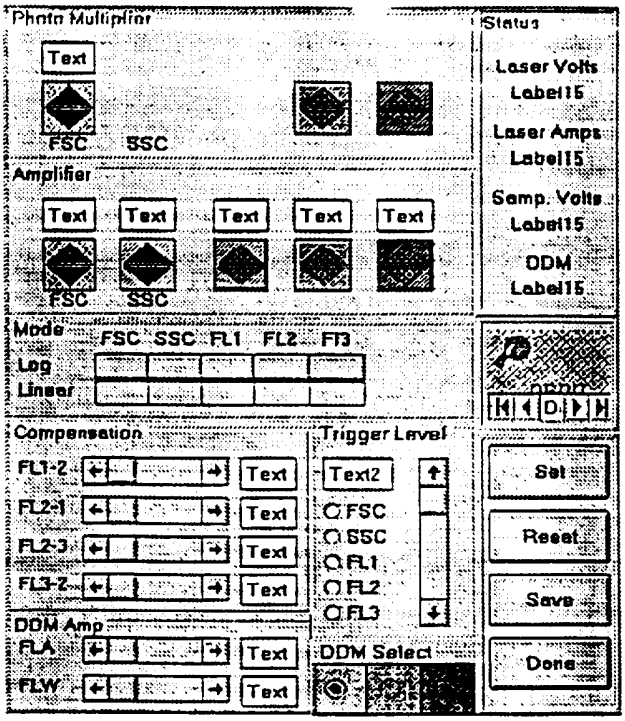
FORM2.FRM - 18

```
      Index           =   4
      Left            =   3360
      LightColor      =   &H000000C0&
      ShadeColor      =   &H00000080&
      ShadowBackColor =   &H000000FF&
      ShadowForeColor =   &H000000FF&
      SpinBackColor   =   &H000000FF&
      SpinForeColor   =   &H00404040&
      TdThickness     =   2
      Top             =   720
      Width           =   495
   End
   Begin SpinButton Spin1
      BackColor       =   &H000080FF&
      Delay           =   100
      ForeColor       =   &H00C0C0C0&
      Height          =   495
      Index           =   3
      Left            =   2640
      LightColor      =   &H000080FF&
      ShadeColor      =   &H00404080&
      ShadowBackColor =   &H000080FF&
      ShadowForeColor =   &H000080FF&
      SpinBackColor   =   &H000080FF&
      SpinForeColor   =   &H00404040&
      TdThickness     =   2
      Top             =   720
      Width           =   495
   End
   Begin TextBox txtNumber
      Height          =   285
      Index           =   0
      Left            =   360
      TabIndex        =   1
      Text            =   "Text1"
      Top             =   360
      Width           =   495
   End
   Begin SpinButton Spin1
      BackColor       =   &H0080FF80&
      ForeColor       =   &H00FFFF00&
      Height          =   495
      Index           =   0
      Left            =   360
      LightColor      =   &H00FFFF80&
      ShadeColor      =   &H00FFFF80&
      ShadowForeColor =   &H0080FF80&
      SpinBackColor   =   &H0080FF80&
      SpinForeColor   =   &H80000008&
      TdThickness     =   1
      Top             =   720
      Width           =   495
   End
   Begin Label Label2
      Alignment       =   2  'Center
      BackStyle       =   0  'Transparent
      Caption         =   "SSC"
      Height          =   255
      Left            =   1080
      TabIndex        =   7
      Top             =   1200
```

FORM2.FRM - 19

```
      Width            =   495
   End
   Begin Label Label1
      Alignment        =   2  'Center
      BackStyle        =   0  'Transparent
      Caption          =   "FSC"
      Height           =   255
      Index            =   0
      Left             =   360
      TabIndex         =   6
      Top              =   1200
      Width            =   495
   End
End
Begin Line Line2
   X1               =   2160
   X2               =   3360
   Y1               =   3120
   Y2               =   3600
End
Begin Line Line1
   X1               =   2160
   X2               =   3360
   Y1               =   3120
   Y2               =   3600
End
End
```


Orbit Diagnostic Operating System
Assay Cube
CubeSel
Specific Assay
AssaySel
Sample Identification
Test Control
Initialize
Start Test
Counts
Machine Control
Calibrate
Manual Adjust
Exit
Cytometer Status
Flow Control
Sheath Fluid
Waste Water
Pressure
Operator Instructions
OK
Results
Clear
Save
Print


Photo Multiplier
Text
FSC
SSC
Amplifier
Text
Text
Text
Text
Text
FSC
SSC
Mode
FSC SSC FL1 FL2 FL3
Log
Linear
Compensation
FL1-2
FL2-1
FL2-3
FL3-2
Text
Text
Text
Text
DDM Amp
FLA
FLW
Text
Text
Trigger Level
Text2
FSC
SSC
FL1
FL2
FL3
DDM Select
Status
Laser Volts
Label15
Laser Amps
Label15
Samp. Volts
Label15
DDM
Label15
Set
Reset
Save
Done

```
FORM1.FRM - 1

Option Explicit


Function vbgenproc ()

End Function

Sub AssaySel_Click ()
Dim y As Integer
Dim x As Integer

'find assay record selected

data2.Refresh
Do While Not (AssaySel.Text = data2.Recordset("assay_name"))
    data2.Recordset.MoveNext
Loop

'get number of events required by this assay

events = data2.Recordset("event_count")

'clear the testdef table;  it holds the name of each token (biomolocule
' assayed) and the base green and over under values

For x = 0 To 1023
For y = 0 To 1
testdef(y, x) = 0
Next y
Next x

datatst.Refresh

'now load the new values for this assay

Do While Not datatst.Recordset.EOF
    If AssaySel.Text = datatst.Recordset("assay") Then
    x = datatst.Recordset(2)   'token value
    tkname(x) = datatst.Recordset("token_name")
    For y = 0 To 1
    testdef(y, x) = datatst.Recordset(y + 4) 'load over under green and base gr
en
    Next y

    End If
    datatst.Recordset.MoveNext

Loop

If x = 0 Then MsgBox "There are no Measurement Parameters defined for this assa
", 48, "Assay Select"
lasttst = x

End Sub

Sub CubeSel_Click ()
Dim y As Integer
Dim x As Integer
data2.Refresh
AssaySel.Clear
Do While Not data2.Recordset.EOF
```

```
FORM1.FRM - 2

    If CubeSel.Text = data2.Recordset("cube_name") Then AssaySel.AddItem data2.
ecordset("assay_name")
    data2.Recordset.MoveNext

Loop

y = ReadPanel(tbuf(0))

loadpbuf
For x = 13 To 42
If pbuf(x) <> tbuf(x) Then y = SendPanel(x, pbuf(x))
If y > 30 Then MsgBox "Flow Cytometer is not responding", 48, "Set Flow Cytomet
r"
Next x
datahld.Refresh
x = 0

Do While Not datahld.Recordset.EOF
    If CubeSel.Text = datahld.Recordset("cube_name") Then
    For y = 0 To 7
    hldtab(y, x) = datahld.Recordset(y + 3)
    Next y
    x = x + 1
    End If
    datahld.Recordset.MoveNext

Loop
If x = 0 Then MsgBox "There are no Classification Parameters defined for this c
be", 48, "Cube Select"
lastnode = x


End Sub

Sub Form_Load ()
Dim x As Integer
'x = InitBrd()
data1.Refresh

Do While Not data1.Recordset.EOF
    CubeSel.AddItem data1.Recordset("cube_name")
    data1.Recordset.MoveNext
Loop

state = 0
grid1.ColWidth(0) = 2400
grid1.ColWidth(1) = 2600
For x = 1 To 63
grid1.RowHeight(x) = 500
Next x
grid1.Row = 0
grid1.Col = 0
grid1.Text = "Biomolecule Assayed"
grid1.Col = 1
grid1.Text = "Result of Assay"
End Sub

Sub MCAdjust_Click ()
If form1!CubeSel.Text <> "" Then Form2.Show

End Sub
```

```
FORM1.FRM - 3


Sub MCCalib_Click ()
OpInst.Text = "Load Calibration Beads into FACS"


End Sub

Sub MCEnd_Click ()
End

End Sub

Sub OpInstOK_Click ()
state = state + 1
Select Case state
Case 0

Case 1
OpInst.Text = "Calibration Complete, Select Cube and Assay"
Case Else
OpInst.Text = ""
TCInit.Enabled = True
End Select

End Sub

Sub ResClear_Click ()
Dim x
For x = 1 To 12
grid1.Row = x
grid1.Col = 0
grid1.Text = ""
grid1.Col = 1
grid1.Text = ""
Next x
grid1.Row = 0
TCStart.Enabled = False

End Sub

Sub TCHalt_Click ()

' during development, the halt button displays the results of classification
' by token, **** token 0 is the reject class

Dim x As Integer
Dim y As Integer
Dim z As Integer
Dim measure As Integer

x = DoTest(lbuf(0, 0), hldtab(0, 0), testdef(0, 0), results(0, 0), events, last
ode, lasttst)

For y = 0 To 1023
If results(2, y) <> 0 Then
            grid1.Row = grid1.Row + 1
            grid1.Col = 0
            grid1.Text = "bead " & y
            grid1.Col = 1
            grid1.Text = results(2, y)
End If
```

FORM1.FRM - 4

```
Next y


End Sub

Sub TCInit_Click ()
Dim x As Integer
Dim y As Integer
'x = InitBrd()
If AssaySel.Text = "" Then
OpInst.Text = "You must select a Cube and Assay first!"
Else
For x = 0 To 1023
For y = 0 To 3
results(y, x) = 0
Next y
Next x

OpInst.Text = "Initialization Complete"
TCStart.Enabled = True
TCHalt.Enabled = True
End If


End Sub

Sub TCStart_Click ()
Dim x As Integer
Dim y As Integer
Dim z As Integer
Dim measure As Integer

x = DoTest(lbuf(0, 0), hldtab(0, 0), testdef(0, 0), results(0, 0), events, last
ode, lasttst)

dataint.Refresh
Do While Not dataint.Recordset.EOF
    If AssaySel.Text = dataint.Recordset("assay") Then
        For x = 0 To 4
        resline(x) = dataint.Recordset(x + 2)'move to temp area
        Next x

        z = resline(0)  'token value
        'test*************************************

        results(2, z) = results(2, z) + 1
        results(0, z) = results(0, z) + 1

        If resline(2) = 0 Then
            measure = results(3, z) / results(2, z) 'sum green over total coun
        Else measure = results(1, z) / results(0, z)'over count divided by und
        End If

        If ((measure >= resline(3)) And (measure <= resline(4))) Then
            grid1.Row = grid1.Row + 1
            grid1.Col = 0
            grid1.Text = tkname(z)
            grid1.Col = 1
            grid1.Text = dataint.Recordset("interpretation")
        End If
```

```
FORM1.FRM - 5

        End If
        dataint.Recordset.MoveNext
    Loop




    graph1.RandomData = 1
    graph1.Refresh
    state = 0
    'AssaySel.Clear
    OpInst.Text = "Test Complete"

    End Sub

    Sub Timer1_Timer ()
    Dim x, y, z  As Integer
    y = ReadPanel(tbuf(0))

        x = tbuf(38)
        ' set Waste Water indicator
        y = x And 1
        z = 1
        If y = 1 Then z = 0
        WWPic(z).Visible = False
        WWPic(y).Visible = True

        ' set Sheath Fluid
        y = (x And 2) / 2
        z = 1
        If y = 1 Then z = 0
        SFPic(z).Visible = False
        SFPic(y).Visible = True

        'set pressure
        y = (x And &H80) / &H80
        z = 1
        If y = 1 Then z = 0
        PRPic(z).Visible = False
        PRPic(y).Visible = True

        'set flow
        y = x And &H70  'mask bits
        Select Case y
        Case &H10
            FCPic(0).Visible = True
            FCPic(1).Visible = False
            FCPic(2).Visible = False
        Case &H20
            FCPic(0).Visible = False
            FCPic(1).Visible = False
            FCPic(2).Visible = True
        Case &H40
            FCPic(0).Visible = False
            FCPic(1).Visible = True
            FCPic(2).Visible = False
        Case 0
            FCPic(0).Visible = False
            FCPic(1).Visible = False
```

```
FORM1.FRM - 6

        FCPic(2).Visible = True
    End Select


End Sub
```

```
FORM2.FRM - 1

Option Explicit
Dim photo(5)  As Integer
Dim ampnum(5) As Integer

Dim mode(5) As Integer
Dim thresh(5) As Integer
Dim fcomp(5) As Integer
Dim trigsav As Integer


Sub Check3D1_Click (Value As Integer)
pbuf(39) = Value

End Sub

Sub Check3D2_Click (Value As Integer)
pbuf(40) = 2 * (Value + 1)

End Sub

Sub setpvals ()
' this sets the manual adjust screen to reflect
' the current values in pbuf

Dim x As Integer
For x = 0 To 4
txtNumber(x) = pbuf(x + 13)
amp(x) = pbuf(x + 18)
Next x

fscmod(pbuf(23)).Value = True
sscmod(pbuf(24)).Value = True
fl1mod(pbuf(25)).Value = True
fl2mod(pbuf(26)).Value = True
fl3mod(pbuf(27)).Value = True

For x = 29 To 33
If pbuf(x) <> 0 Then
trigval = pbuf(x)
trigger(x - 29).Value = True
End If
Next x

For x = 0 To 3
text1(x) = pbuf(x + 34)
Next x

ddmtxt(0) = pbuf(41)
ddmtxt(1) = pbuf(42)

If pbuf(40) <> 0 Then option1(pbuf(40) - 2).Value = True

stat(0).Caption = Format$(pbuf(10) * .05, "###.00")
stat(1).Caption = Format$(pbuf(11) * .02, "###.00")
stat(2).Caption = Format$(pbuf(12) / 100, "###.00")

If pbuf(39) = 1 Then
stat(3).Caption = "Enabled"
Else stat(3).Caption = "Disabled"
End If
```

FORM2.FRM - 2

```
End Sub

Sub amp_Change (Index As Integer)
If Val(amp(Index).Text) > 999 Then amp(Index).Text = "999"
pbuf(Index + 18) = Val(amp(Index).Text)
amp(Index).Text = Format(pbuf(Index + 18))

End Sub

Sub Command3D1_Click ()
form2.Hide
End Sub

Sub Command3D2_Click ()
Dim x As Integer
Dim y As Integer
Dim z As Integer
y = ReadPanel(tbuf(0))
For x = 13 To 42
If pbuf(x) <> tbuf(x) Then y = SendPanel(x, pbuf(x))
Next x
End Sub

Sub Command3D3_Click ()
Dim y As Integer
Dim x As Integer
loadpbuf
setpvals
For x = 13 To 42
y = SendPanel(x, pbuf(x))
If y > 30 Then MsgBox "Flow Cytometer is not responding", 48, "Set Flow Cytomet
r"
Next x

End Sub

Sub Command3D4_Click ()
savepbuf
End Sub

Sub Command3D5_Click ()
form2.Hide

End Sub

Sub ddmscroll_Change (Index As Integer)
pbuf(Index + 41) = ddmscroll(Index).Value
ddmtxt(Index) = Format(ddmscroll(Index))

End Sub

Sub ddmtxt_Change (Index As Integer)
If Val(ddmtxt(Index)) > 999 Then ddmtxt(Index) = "999"
ddmscroll(Index).Value = Val(ddmtxt(Index))
pbuf(Index + 41) = ddmscroll(Index).Value

End Sub
```

```
FORM2.FRM - 3

Sub fl1mod_Click (Index As Integer, Value As Integer)
pbuf(25) = Index
If Index = 0 Then
    spina(2).Enabled = False
    amp(2).Enabled = False
Else
    spina(2).Enabled = True
    amp(2).Enabled = True
End If

End Sub

Sub fl2mod_Click (Index As Integer, Value As Integer)
pbuf(26) = Index
If Index = 0 Then
    spina(3).Enabled = False
    amp(3).Enabled = False
Else
    spina(3).Enabled = True
    amp(3).Enabled = True
End If

End Sub

Sub fl3mod_Click (Index As Integer, Value As Integer)
pbuf(27) = Index
If Index = 0 Then
    spina(4).Enabled = False
    amp(4).Enabled = False
Else
    spina(4).Enabled = True
    amp(4).Enabled = True
End If

End Sub

Sub Form_Load ()
Dim x As Integer
Dim y As Integer
loadpbuf
setpvals
form2.Caption = form1!CubeSel.Text & " Cube Manual Adjust"


End Sub

Sub fscmod_Click (Index As Integer, Value As Integer)
pbuf(23) = Index
If Index = 0 Then
    spina(0).Enabled = False
    amp(0).Enabled = False
Else
    spina(0).Enabled = True
    amp(0).Enabled = True
End If



End Sub

Sub HScroll1_Change (Index As Integer)
```

FORM2.FRM - 4

```
pbuf(Index + 34) = HScroll1(Index).Value
text1(Index) = Format(HScroll1(Index))
End Sub

Sub Option1_Click (Index As Integer)
pbuf(40) = Index + 2
pbuf(39) = 1
End Sub

Sub Spin1_SpinDown (Index As Integer)
Dim min
pbuf(Index + 13) = pbuf(Index + 13) - 1
If Index = 0 Then min = 0 Else min = 150
If pbuf(Index + 13) < min Then pbuf(Index + 13) = min
txtNumber(Index).Text = Format(pbuf(Index + 13))
End Sub

Sub Spin1_SpinUp (Index As Integer)
Dim max
pbuf(Index + 13) = pbuf(Index + 13) + 1
If Index = 0 Then max = 4 Else max = 999
If pbuf(Index + 13) > max Then pbuf(Index + 13) = max
txtNumber(Index).Text = Format(pbuf(Index + 13))

End Sub

Sub Spina_SpinDown (Index As Integer)
Dim min
pbuf(Index + 18) = pbuf(Index + 18) - 1
min = 100
If pbuf(Index + 18) < min Then pbuf(Index + 18) = min
amp(Index).Text = Format(pbuf(Index + 18))

End Sub

Sub Spina_SpinUp (Index As Integer)
Dim max
pbuf(Index + 18) = pbuf(Index + 18) + 1
max = 999
If pbuf(Index + 18) > max Then pbuf(Index + 18) = max
amp(Index).Text = Format(pbuf(Index + 18))

End Sub

Sub sscmod_Click (Index As Integer, Value As Integer)
pbuf(24) = Index
If Index = 0 Then
    spina(1).Enabled = False
    amp(1).Enabled = False
Else
    spina(1).Enabled = True
    amp(1).Enabled = True
End If

End Sub

Sub Text1_Change (Index As Integer)
If Val(text1(Index)) > 999 Then text1(Index) = "999"
HScroll1(Index).Value = Val(text1(Index))
pbuf(Index + 34) = HScroll1(Index).Value
```

```
FORM2.FRM - 5

End Sub

Sub trigger_Click (Index As Integer, Value As Integer)
Dim x
For x = 29 To 33
pbuf(x) = 0
Next x
pbuf(Index + 29) = Val(trigval.Text)
trigsav = Index
pbuf(28) = 128 + Index
End Sub

Sub trigval_Change ()
If Val(trigval.Text) > 999 Then trigval.Text = "999"
VScroll1.Value = Val(trigval.Text)
pbuf(trigsav + 29) = VScroll1.Value

End Sub

Sub VScroll1_Change ()
trigval.Text = Format(VScroll1.Value)
pbuf(trigsav + 29) = VScroll1.Value

End Sub
```

MODULE1.BAS - 1

```
Option Explicit
Global pbuf(64) As Integer
Global tbuf(64) As Integer

Global lbuf(8, 32) As Integer
Global hldtab(7, 1023) As Integer
Global results(3, 1023) As Long
Global testdef(1, 1023) As Integer
Global resline(5) As Integer
Global tkname(1024) As String
Global state As Integer
Global lastnode As Integer
Global lasttst As Integer
Global events As Long

Declare Function DoTest Lib "c:\msvc\bin\orbit.dll" (lbuf As Integer, hldtab As
Integer, testdef As Integer, results As Long, ByVal events&, ByVal lastnode%, B
Val lasttst%) As Integer
Declare Function InitBrd Lib "c:\msvc\bin\orbit.dll" () As Integer
Declare Function SendPanel Lib "c:\msvc\bin\orbit.dll" (ByVal parm%, ByVal panv
l%) As Integer
Declare Function ReadPanel Lib "c:\msvc\bin\orbit.dll" (pbuf As Integer) As Int
ger
Declare Function ReadList Lib "c:\msvc\bin\orbit.dll" (lbuf As Integer) As Inte
er


Sub loadpbuf ()
' for a selected cube, the data base values are loaded into pbuf

Dim x As Integer
form2!facset.Refresh
Do While form2!facset.Recordset.EOF = False
If form1!CubeSel.Text = form2!facset.Recordset(0) Then Exit Do
form2!facset.Recordset.MoveNext
Loop

For x = 0 To 42
pbuf(x) = form2!facset.Recordset(x + 1)
Next x

End Sub

Sub savepbuf ()
' This saves the current values in pbuf to the data base

Dim x As Integer
form2!facset.Refresh
Do While form2!facset.Recordset.EOF = False
If form1!CubeSel.Text = form2!facset.Recordset(0) Then Exit Do
form2!facset.Recordset.MoveNext
Loop
form2!facset.Recordset.Edit
For x = 0 To 42
form2!facset.Recordset(x + 1) = pbuf(x)
Next x
form2!facset.Recordset.Update
End Sub
```

```
MODULE2.BAS - 1
Option Explicit
```

```
////////////////////////////////////////////////////////////
//
//  MDXDLL.DLL source.
//
////////////////////////////////////////////////////////////

#include <windows.h>
#include "mdxdll.h"

int WINAPI _export InitBrd(void)
{
__asm
        {
        mov     dx,brdctrl
        mov     ax,20h
        out     dx,ax
        mov     dx,brdctrl
        in      ax,dx
        or      ax,outfifoclr   ;set both fifos to clear
        or      ax,infifoclr
        out     dx,ax

        mov     dx,inpxfer
        mov     ax,0    ;set xfer count to 0
        out     dx,ax

        mov     dx,prtctrl
        mov     ax,0
        or      ax,ctl0 ;set control lines to idle
        or      ax,ctl1
        out     dx,ax


        mov     dx,prtctrl
        in      ax,dx
        mov     bx,preset       ;reset facs
        not     bx
        and     ax,bx
        out     dx,ax

        or      ax,preset       ;toggle
        out     dx,ax

        mov     dx,brdsts
        in      ax,dx

        mov     dx,inpxfer
        mov     ax,0
        out     dx,ax
        mov     dx,prtctrl
        mov     ax,6    ;assert ctl0 &1
        out     dx,ax

        // asm code here

        }

  //SendPanel(ddmena,1);
  return(0);
  }

  int WINAPI _export SendPanel(int parm, int PanVal)
```

```
{
int i = 0;

__asm
        {
        mov     dx,brdctrl
        in      ax,dx
        mov     bx,inen
        not     bx
        and     ax,bx
        or      ax,outen
        out     dx,ax

        mov     dx,prtctrl
        in      ax,dx
        mov     bx,ctl0
        not     bx
        and     ax,bx
        out     dx,ax

        mov     dx,brdctrl
        in      ax,dx
        or      ax,outfifoclr
        out     dx,ax
        mov     bx,outfifoclr
        not     bx
        and     ax,bx
        out     dx,ax

        mov     dx,bdata
        mov     ax,1
        out     dx,ax

        mov     bx,parm         ;get code
        shl     bx,10
        mov     ax,PanVal       ;get panel value
        and     ax,3ffh
        or      ax,bx
        out     dx,ax    ;send it out

        mov     dx,dlyctrl
        mov     ax,0160h
        out     dx,ax

        mov     dx,brdctrl
        in      ax,dx
        or      ax,hshken
        out     dx,ax
        mov     cx,4000

wsend:  mov     dx,brdsts
        in      ax,dx
        mov     bx,ax
        and     bx,1     ;1=empty fifo
        loope   wsend
        and     ax,40h  ;bit 6 = 1 = xfer pending
        jnz         wsend
        mov     dx,prtctrl
        in      ax,dx
        or      ax,ctl0
        out     dx,ax
```

```
        mov     dx,brdctrl
        in      ax,dx
        mov     bx,outen
        or      bx,hshken
        not     bx
        and     ax,bx
        out     dx,ax

        mov     dx,dlyctrl
        mov     ax,0101h
        out     dx,ax
        // asm code here

        }
return(i);

}


int WINAPI _export ReadPanel(int __far *pbuf)
{
int far* pbufptr = pbuf;

__asm
        {
        mov     dx,brdctrl
        in      ax,dx
        or      ax,outfifoclr   ;set both fifos to clear
        or      ax,infifoclr
        out     dx,ax

        mov     dx,inpxfer
        mov     ax,0    ;set xfer count to 0
        out     dx,ax

        mov     dx,prtctrl
        mov     ax,0
        or      ax,ctl0 ;set control lines to idle
        or      ax,ctl1
        out     dx,ax


        mov     dx,prtctrl
        in      ax,dx
        or      ax,preset       ;toggle
        out     dx,ax

lowlp:  mov     dx,prtsts
        in      ax,dx
        mov     bx,ax
        and     ax,stsl ;has stsl gone low yet?
        jz      waithigh        ;yes, wait for it to go back high
        jmp     lowlp   ;***************

waithigh:

        mov     dx,brdctrl      ;no, panel coming so set up read
        in      ax,dx
        and     al,0eeh ;handsk off out off
        or      ax,8    ;clr in fifo
        out     dx,ax
```

```
        and     al,0f7h  ;fifo clr off
        out     dx,ax

        mov     dx,inpxfer      ;set up xfer count
        mov     ax,43
        out     dx,ax

        mov     dx,prtctrl
        in      ax,dx
        mov     bx,ctl0
        not     bx
        and     ax,bx
        out     dx,ax   ;set ctl0 to request panel data

        mov     dx,dlyctrl
        mov     ax,101h
        out     dx,ax

        mov     dx,brdctrl
        in      ax,dx
        or      ax,hshken       ;initiate handshake
        out     dx,ax

waitdata:
        mov     dx,brdsts
        mov     bx,xferincom
        or      bx,xferpend
        in      ax,dx
        and     ax,bx   ;wait until xfer not pending
        jnz     waitdata        ;or incomplete

        mov     dx,prtctrl  ;done
        in      ax,dx
        or      ax,ctl0 ;stop
        out     dx,ax
        mov     dx,brdctrl
        in      ax,dx
        and     al,0ebh ;turn off handshake and input enable
        out     dx,ax
        push    di
        push    es
        mov     cx,43
        les     di,pbufptr
        mov     dx, bdata
readlp:
        in      ax,dx   ;read data from fifo
        mov     bx,ax
        and     ax,3ffh
        and     bx,0fc00h
        shr     bx,9    ;right 10 * 2
        mov     word ptr es:[di+bx],ax
        loop    readlp
        pop             es
        pop             di

        }
return(0);
}

int WINAPI _export DoTest(int __far *lbuf, int __far *hldtab,
                          int __far *testdef, long __far *results,
                          long events, int lastnode, int lasttst)
```

```
{

int far* lbufptr = lbuf;
int far* hldptr = hldtab;
int far* testptr = testdef;
long far* resptr = results;

int x,y,z,bdi,goodcnt,token = 0;

while (events > 0)
        {
        goodcnt = ReadList(lbufptr);
        if (goodcnt > 0)
                {
                for (bdi = 0; bdi < goodcnt; bdi++)
                        {
                        x = y = 0;
                        // orange correction goes here for lbufptr[bdi][fl2]
                        // x is the current node in the hld table
                        token = -1;
                        while (token < 0)
                                {
                                z = lbufptr[bdi * 8 + (hldptr[x * 8])];
                                // z is the value of the parameter under test
                                if ((z >= hldptr[x * 8 + 2]) && (z <= hldptr[x * 8 + 3])) //low
val && high val
                                        {
                                        if (hldptr[x * 8 + 4] == 0) token = hldptr[x * 8 + 6]; /
/ 0 means done, get token true
                                        else x = hldptr[x * 8 + 4]; // get node true
                                        }
                                else
                                        {
                                        if (hldptr[x * 8 + 5] == 0) token = hldptr[x * 8 + 7];
/ get token false
                                        else x = hldptr[x * 8 + 5]; // get node false
                                        }
                                }
                                events--;
                                z = lbufptr[bdi * 8 + 2]; // z is FL1
                                if (z < testptr[token * 2 + 1]) resptr[token * 4]++; //inc unde
 count
                                if (z > testptr[token * 2 + 1]) resptr[token * 4 + 1]++; //inc
ver count
                                resptr[token * 4 + 2]++;  // inc total count
                                resptr[token * 4 + 3] += z;  //sum FL1
                        }
                }

        }


__asm
        {
         nop
        }

return(0);
}


int WINAPI _export ReadList(int __far *lbuf)
```

```
{
int far* lbufptr = lbuf;
int i = 0;

__asm
        {
        ;*****************************************************************
        ;               read list mode
        ;*****************************************************************

        mov     dx,brdctrl
        in      ax,dx
        or      ax,outfifoclr   ;set both fifos to clear
        or      ax,infifoclr
        out     dx,ax

        mov     dx,inpxfer
        mov     ax,0    ;set xfer count to 0
        out     dx,ax

        mov     dx,prtctrl
        mov     ax,0
        or      ax,ctl0 ;set control lines to idle
        or      ax,ctl1
        out     dx,ax


        mov     dx,prtctrl
        in      ax,dx
        or      ax,preset       ;toggle
        out     dx,ax

lowlp1: mov     dx,prtsts
        in      ax,dx
        mov     bx,ax
        and     ax,sts0 ;has sts0 gone low yet?
        jz      waithigh1       ;yes, wait for it to go back high
        jmp     lowlp1  ;  ********

waithigh1:

        mov     dx,brdctrl      ;no, list mode coming so set up read
        in      ax,dx
        and     al,0eeh ;handsk off out off
        or      ax,8    ;clr in fifo
        out     dx,ax

        and     al,0f7h  ;fifo clr off
        out     dx,ax

        push    es
        push    di
        push    ds
        push    si
        les     di,lbufptr


        mov     dx,inpxfer      ;set up xfer count
        mov     ax,120  ;7 vals + chk sum * 15 events
        out     dx,ax

        mov     dx,prtctrl
        in      ax,dx
```

```
        mov     bx,ctl1
        not     bx
        and     ax,bx
        out     dx,ax   ;set ctl1 to request list data

        mov     dx,dlyctrl
        mov     ax,101h
        out     dx,ax

        mov     dx,brdctrl
        in      ax,dx
        or      ax,hshken       ;initiate handshake
        out     dx,ax

waitdata1:
        mov     dx,brdsts
        mov     bx,xferincom
        or      bx,xferpend
        in      ax,dx
        and     ax,bx   ;wait until xfer not pending
        jnz     waitdata1       ;or incomplete
        mov     dx,bdata
        mov     cx,120  ;number of words to read
chunk:  in      ax,dx
        stosw
        loop    chunk
        mov     dx,prtctrl  ;done
        in      ax,dx
        or      ax,ctl1 ;stop
        out     dx,ax
        mov     dx,brdctrl
        in      ax,dx
        and     al,0ebh ;turn off handshake and input enable
        out     dx,ax


        les     di,lbufptr
        lds     si,lbufptr
        mov     cx,15   ;up to 15 good events could be present

        ;check alignment

        mov     dx,0    ;count of good records


        test    word ptr [si],0e000h
        jz      alnok
        dec     cx      ;only 14 max possible now
        push    cx
        mov     cx,7    ;seven other possible alignments
alnlp:  lodsw
        test    ax,0e000h
        jz      nowalnok        ;now have alignment
        loop    alnlp
        pop     cx
        jmp     badbuff
nowalnok:
        sub     si,2    ;repoint to good align
        pop     cx      ;restore loop count
alnok:  push    cx
        mov     cx,7
        mov     bx,0
sumchk: lodsw
```

```
        add     bx,ax
        loop    sumchk
        lodsw
        cmp     bx,ax   ;is check sum good
        jne     nocopy
        sub     si,16   ;repoint to start of rec
        mov     cx,8
clncpy: lodsw
        and     ax,3ffh ;knock off parm number
        stosw
        loop    clncpy
        inc     dx      ;count good records
nocopy: pop     cx
        loop    alnok
badbuff:
        mov     i,dx

        pop     si
        pop     ds
        pop     di
        pop     es




        }
return(i);
}


////////////////////////////////////////////////////////
//
//  DLL Initialization and exit.
//
int WINAPI LibMain(HANDLE hInst, WORD wDataSeg,
                   WORD cbHeapSize, LPSTR lpszCmdLine)
{
  hModInst = hInst;
  if (cbHeapSize != 0)
    UnlockData(0);
  return 1;
}


int _export WINAPI WEP(int nParam)
{
  return 1;
}
```

```
//////////////////////////////////////////////////////////////
//
//  Include file for MDXDLL.DLL
//
//////////////////////////////////////////////////////////////

#ifdef __cplusplus
extern "C" {
#endif


//////////////////////////////////////////////////////////////
//
// Define DEBUG macros.
//
#if defined(_DEBUG) && !defined(_AFX)

char _sz_ASSERT[255];

#define ASSERT(a)  if(!(a)) { wsprintf(_sz_ASSERT,  \
   "assertion failed in file %s at line %d\r\n",      \
   (LPSTR)(__FILE__),__LINE__); OutputDebugString(_sz_ASSERT); }
#define TRACE(a)   OutputDebugString(a"\r\n")
#define TRACE1(a,b)    { wsprintf(_sz_ASSERT,a"\r\n",(int)(b)); \
   OutputDebugString(_sz_ASSERT); }
#define TRACE2(a,b,c)  { wsprintf(_sz_ASSERT,a"\r\n",(int)(b),(int)(c)); \
   OutputDebugString(_sz_ASSERT); }

#endif

#if !defined(_DEBUG) && !defined(_AFX)

#define ASSERT(a)
#define TRACE(a)
#define TRACE1(a,b)
#define TRACE2(a,b,c)

#endif


//////////////////////////////////////////////////////////////
//
// From Windowsx.h
//
#define     GlobalPtrHandle(lp)          \
            ((HGLOBAL)LOWORD(GlobalHandle(SELECTOROF(lp))))

#define     GlobalLockPtr(lp)       \
            ((BOOL)SELECTOROF(GlobalLock(GlobalPtrHandle(lp))))
#define     GlobalUnlockPtr(lp)     \
            GlobalUnlock(GlobalPtrHandle(lp))

#define     GlobalAllocPtr(flags, cb)   \
            (GlobalLock(GlobalAlloc((flags), (cb))))
#define     GlobalReAllocPtr(lp, cbNew, flags)  \
            (GlobalUnlockPtr(lp), GlobalLock(GlobalReAlloc(GlobalPtrHandle(lp) , (cbNew), (
lags))))
#define     GlobalFreePtr(lp)       \
            (GlobalUnlockPtr(lp), (BOOL)GlobalFree(GlobalPtrHandle(lp)))

//////////////////////////////////////////////////////////////
```

```
//
// Global variables.
//

HINSTANCE hModInst;     // module handle.


// Cytomation board definitions

#define base    0x240
#define bdata   base + 0
#define brdctrl base + 2
#define brdsts  base + 4
#define prtctrl base + 6
#define prtsts  base + 8
#define dlyctrl base + 0x0a
#define inpxfer base + 0x0c

        //facs codes

#define ddmena  39

        //board control

#define outen   1
#define outfifoclr  2
#define inen    4
#define infifoclr   8
#define hshken      0x10

        //board status

#define outfifoe    1
#define outfifoh    2
#define outfifof    4
#define infifoe     8
#define infifoh     0x10
#define infifof     0x20
#define xferpend    0x40
#define xferincom   0x80

        //port control

#define preset      1
#define ctl0        2
#define ctl1        4

        //port status

#define eir         1
#define psts        2
#define sts0        4
#define sts1        8


////////////////////////////////////////////////////////
//
// Exported Functions.
//


////////////////////////////////////////////////////////
```

```
//
// Local Functions.
//

int WINAPI _export SendPanel(int parm, int PanVal);
int WINAPI _export ReadPanel(int pbuf[]);
int WINAPI _export ReadList(int lbuf[]);
int WINAPI _export InitBrd (void);
int WINAPI _export DoTest(int lbuf[], int hldtab[],
                          int testdef[], long results[],
                          long events, int lastnode, int lasttst);

#ifdef __cplusplus
}
#endif
```

```
;-----------------------------------------------------------
;
; Module Definition file for MDXDLL.DLL
;
;-----------------------------------------------------------
LIBRARY    MDXDLL

DESCRIPTION  'MDXDLL Library'

EXETYPE    WINDOWS

CODE       PRELOAD MOVABLE
DATA       PRELOAD MOVABLE

HEAPSIZE   1024

EXPORTS
  WEP                  @1 RESIDENTNAME
  InitBrd              @2
  SendPanel            @3
  ReadPanel            @4
  ReadList             @5
  DoTest               @6
```

WHAT IS CLAIMED IS:

1. A method of preparing a beadset capable of detecting a plurality of analytes in a single fluid sample by flow cytometric analysis comprising:

(a) obtaining a plurality of subsets of beads wherein the beads in each subset are sufficiently homogeneous with respect to at least three selected classification parameters (C_1, C_2, C_3 ... C_n) and sufficiently different in at least one of said classification parameters from beads in any other subset so that the profile of classification parameter values within each subset detectable by flow cytometry is unique;

(b) coupling the beads within each subset to a reactant that will specifically react with a given analyte of interest in a fluid to be tested; and

(c) mixing the subsets of beads to produce a beadset, wherein the subset identity and therefore the reactant to which the bead has been coupled is identifiable by flow cytometry based on the unique classification parameter profile of the beads.

2. A beadset capable of detecting a plurality of analytes in a single fluid sample by flow cytometric analysis comprising a plurality of subsets of beads wherein:

(a) the beads in each subset are sufficiently homogeneous with respect to at least three selected classification parameters (C_1, C_2, C_3 ... C_n) and sufficiently different in at least one of said classification parameters from beads in any other subset so that the profile of classification parameter values within each subset detectable by flow cytometry is unique;

(b) wherein the beads within each subset are coupled to a reactant that will specifically react with a given analyte of interest in a fluid to be tested; and

(c) wherein said subsets have been mixed to produce the beadset, characterized in that the subset identity and therefore the reactant to which the bead has been coupled is identifiable based on the unique classification parameter profile of the bead.

3. A method of flow cytometric analysis capable of detecting a plurality of analytes of interest in a single fluid sample comprising:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to a reactant that will specifically react with a selected analyte of interest in a fluid to be tested;

(b) mixing, to produce a reacted bead sample, the beadset with the fluid to be tested under conditions that will allow reactions between analytes of interest in the fluid and the reactants on the beads in said set, wherein a reaction between a reactant

and an analyte of interest on a bead causes a change in the value of a fluorescent signal (F_m) emitted from said bead;

(c) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(d) identifying the subset to which each bead belongs and therefore the reactant on the bead as a function of the unique profile of classification parameter values; and

(e) detecting the presence or absence of a particular analyte of interest in said sample as a function of the identification in step (d) and a change in the F_m values of the beads in each of said subsets in the reacted fluid sample relative to the F_m values of the beads in each of said subsets not reacted with said fluid.

4. A method of flow cytometric analysis capable of detecting a plurality of analytes of interest in a single fluid sample comprising:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to a reactant that will specifically react with a selected analyte of interest in a fluid to be tested;

(b) mixing, to produce a reacted bead sample, the beadset with the fluid to be tested under conditions that will allow reactions between analytes of interest in the fluid and the reactants on the beads in said set;

(c) mixing with the reacted bead sample a fluorescent label under conditions such that said label will bind to and thereby increase the value of a fluorescent signal F_m emitted from said bead;

(d) analyzing the reacted sample containing the fluorescent label by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(e) identifying the subset to which each bead belongs and therefore the reactant on the bead as a function of the unique profile of classification parameter values; and

(f) detecting the presence or absence of a particular analyte of interest in said sample as a function of the identification in step (e) and an increase in the F_m values of the beads in each of said subsets in the reacted fluid sample relative to the F_m values of the beads in each of said subsets not reacted with said fluid.

5. A method of flow cytometric analysis capable of detecting a plurality of analytes of interest in a single sample comprising:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently different from beads in any other subset in at least one of said

classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique,

(2) are coupled to a reactant that will specifically react with a selected analyte of interest in a fluid to be tested, and

(3) are reacted with a fluorescently labeled compound which competes with said analyte for reaction with said reactant;

(b) mixing, to produce a reacted bead sample, the beadset with the fluid to be tested under conditions that will allow reactions between analytes of interest in the fluid and the reactants on the beads in said set and thereby to allow the analytes to competitively inhibit or displace the fluorescently labeled compounds from said beads, resulting in a decrease in a fluorescent signal F_m emitted from a bead with which an analyte of interest in the fluid has reacted;

(c) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(d) identifying the subset to which each bead belongs and therefore the reactant on the bead as a function of the unique profile of classification parameter values; and

(e) detecting the presence or absence of a particular analyte of interest in said sample as a function of the identification in step (d) and an increase in the F_m values of the beads in each of said subsets in the reacted fluid sample relative to the F_m values of the beads in each of said subsets not reacted with said fluid.

6. The method of any one of claims 3, 4, and 5 wherein C_1, C_2, and C_3 are each different and are selected from the group consisting of forward light scatter, side light scatter and fluorescence.

7. The method of any one of claims 3, 4, and 5 wherein n is greater than or equal to 4 and C_1 is forward angle light scatter, C_2 is side angle light scatter, C_3 is fluorescence at a first wavelength and C_4 is fluorescence at a second wavelength.

8. The method of claim 7 wherein said first wavelength is red and said second wavelength is orange.

9. The method of claim 7 wherein said first wavelength is red, said second wavelength is orange, and the wavelength of said F_m signal is green.

10. The method of claim 3 wherein said analytes of interest are antigens and said reactants are antibodies specifically reactive with said antigens.

11. The method of claim 3 wherein said analytes of interest are antibodies and said reactants are antigens specifically reactive with said antibodies.

12. The method of claim 3 wherein said analytes of interest are antigens selected from the group consisting of bacterial, viral, fungal, mycoplasmal, rickettsial, chlamydial and protozoal antigens and said reactants are antibodies specifically reactive with said antigens.

13. The method of claim 3 wherein said reactants are antigens selected from the group consisting of bacterial, viral, fungal, mycoplasmal, rickettsial, chlamydial and protozoal

antigens and said analytes of interest are antibodies specifically reactive with said antigens.

14. The method of any one of claims 10 or 12 wherein said antigens are antigens borne by pathogenic agents responsible for sexually transmitted disease.

15. The method of any of claims 10 or 12 wherein said antigens are antigens borne by pathogenic agents responsible for a pulmonary disorder.

16. The method of any of claims 10 or 12 wherein said antigens are antigens borne by pathogenic agents responsible for a gastrointestinal disorder.

17. The method of claim 3 wherein said analytes of interest are substances of abuse.

18. The method of claim 3 wherein said analytes of interest are therapeutic drugs.

19. The method of claim 3 wherein said analytes of interest are antigens or antibodies associated with one or more selected pathological syndromes.

20. The method of claim 19 wherein said syndromes are selected from the group consisting of malignancy, allergy, autoimmune diseases, and blood borne viruses.

21. The method of claim 19 wherein at least one said syndrome is a cardiovascular disorder.

22. The method of claim 3 wherein said analytes of interest are selected from the group consisting of analytes testing for pregnancies and hormones.

23. The method of claim 3 wherein said fluorescent signal is emitted from fluoresceinated antibodies specific for antibodies coupled to said beads in said set.

24. The method of claim 3 wherein said fluorescent signal is emitted from a fluoresceinated compound specifically reactive with an immunoglobulin molecule.

25. The method of claim 3 wherein said fluorescent signal is emitted from an agent selected from the group consisting of a fluoresceinated anti-immunoglobulin antibody or a specifically reactive fragment thereof, fluoresceinated protein A, and fluoresceinated protein G.

26. The method of claim 19 wherein said analyte comprises autoantibodies and said antigens comprise oligopeptide epitopes reactive with said autoantibodies, said fluorescent labels comprise fluorescent monoclonal antibodies reactive with said epitopes and wherein the presence of the analyte autoantibodies is detected as a result in a decrease of F_m.

27. The method of claim 3 wherein said analytes are enzymes, said reactants are fluorescently labeled substrates for said enzymes, said change in F_m results from cleavage of said substrates from said beads.

28. The method of claim 27 wherein said enzymes are selected from the groups consisting of proteases, glycosidases, nucleotidases, oxidoreductases, hydrolyases, esterases, convertases, ligases, transferases, phosphorylases, lyases, lipases, peptidases, dehydrogenases, oxidases, phospholipases, decarboxylases, invertases, aldolases, transaminases, synthetases, and phosphotases.

29. The method of claim 3 wherein the fluid to be tested is selected from the group consisting of plasma, serum, tears, mucus, saliva, urine, pleural fluid, spinal fluid and gastric fluid, sweat, semen, vaginal secretions, fluid from ulcers and other surface eruptions, blisters, and abscesses, and extracts of tissues including biopsies of normal, malignant, and suspect tissues.

30. A method of flow cytometric analysis for detection of immunoglobulins in a fluid sample comprising the steps of:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to an immunoglobulin that corresponds to the immunoglobulin to be assayed for in the fluid sample;

(b) obtaining a fluorescently labeled immunoglobulin-binding reagent capable of reacting with the immunoglobulins to be detected;

(c) mixing, to produce a reacted bead sample, the beadset with the fluid sample to be tested and the fluorescently labeled immunoglobulin-binding reagent under conditions that will allow competitive binding reactions between the

immunoglobulin-binding reagent and immunoglobulin in the fluid to be tested and between the immunoglobulin-binding reagent and the immunoglobulin on the beads in said set, wherein a reaction between a bead-bound immunoglobulin and the fluorescently labeled immunoglobulin-binding reagent causes an increase in the value of a fluorescent signal (F_m) emitted from said bead;

(d) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(e) identifying the subset to which each bead belongs and therefore the immunoglobulin on the bead as a function of the unique profile of classification parameter values; and

(f) detecting a corresponding immunoglobulin in said sample as a function of the identification in step (e) and a change in the F_m values of the beads in each of said subsets in the reacted fluid sample relative to the F_m values of the beads in each of said subsets not reacted with said fluid.

31. The method of claim 30 wherein said immunoglobulins to be detected are immunoglobulins belonging to different immunoglobulin classes.

32. The method of claim 31 wherein said classes are selected from the group consisting of IgG, IgM, IgA, and IgE.

33. The method of claim 32 wherein said immunoglobulins to be detected are immunoglobulins belonging to different immunoglobulin sub-classes.

34. The method of claim 33 wherein said subclasses are selected from the group consisting of human IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2.

35. A method of flow cytometric analysis for detection of immunoglobulin specific for a particular epitope of interest in a sample comprising the steps of:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to a monoclonal antibody preparation which is specific for an epitope that is the same epitope as that epitope which binds to an immunoglobulin to be assayed for;

(b) obtaining a plurality of fluorescently labeled reagents wherein each of said reagents bears an epitope to which the monoclonal antibody preparation coupled to the beads within a subset binds;

(c) mixing, to produce a reacted bead sample, the beadset with the fluid sample to be tested and the fluorescently labeled reagents under conditions that will allow competitive binding reactions between the fluorescently labeled reagents and immunoglobulin in the fluid to be tested and between the fluorescently labeled

reagents and the monoclonal antibodies on the beads wherein a reaction between a bead-bound antibody and the fluorescently labeled reagent causes an increase in the value of a fluorescent signal (F_m) emitted from said bead;

(d) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(e) identifying the subset to which each bead belongs and therefore the monoclonal antibody on the bead as a function of the unique profile of classification parameter values; and

(f) detecting the presence or absence of an immunoglobulin in said sample specific for said particular epitope as a function of the identification in step (e) and a change in the F_m values of the beads in each of said subsets in the reacted fluid sample relative to the F_m. values of the beads in each of said subsets not reacted with said fluid.

36. The method of claim 35 wherein the epitopes are epitopes located on viral antigens.

37. The method of claim 36 wherein said viral antigen is an antigen from HIV.

38. A method of flow cytometric analysis for detection of analytes commonly elevated in pregnancy in a fluid sample comprising the steps of:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently

different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to an antibody which is specific for an analyte to be assayed for in the fluid sample;

(b) obtaining a plurality of preparations of antibody molecules wherein each of said preparations contains fluorescently labeled antibodies specific for an analyte to be assayed for in the fluid sample;

(c) mixing, to produce a reacted bead sample, the beadset with the fluid sample to be tested and the fluorescently labeled antibodies under conditions that will allow binding reactions between the antibody that is coupled to the bead, the analyte of interest in the fluid to be tested, and the fluorescently labeled antibodies so as to bind said fluorescent antibodies to said beads though binding to said enzymes which are in turn bound to said beads though said bead-bound antibodies and wherein a bridging reaction between a bead-bound antibody, the analyte to which that antibody binds, and the fluorescently labeled antibody specific for said enzyme causes an increase in the value of a fluorescent signal (F_m) emitted from said bead;

(d) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(e) identifying the subset to which each bead belongs and therefore the antibody on the bead as a function of the unique profile of classification parameter values; and

(f) detecting the analyte in said sample as a function of the identification in step (e) and a change in the F_m values of the beads in each of said subsets in the reacted fluid sample relative to values of the beads in each of said subsets not reacted with said fluid.

39. The method of claim 38 wherein said analytes are selected from the group consisting of human chorionic gonadotropin, alpha fetoprotein, and 3' estradiol.

40. A method of flow cytometric analysis for determining the epitope to which a monoclonal antibody binds comprising the steps of:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to a peptide which provides a given epitope;

(b) obtaining a fluorescently labeled monoclonal antibody of interest;

(c) mixing, to produce a reacted bead sample, the beadset with the fluorescently labeled monoclonal antibody under conditions that will allow binding reactions

between the bead-bound peptide which provides the epitope to which the monoclonal antibody is capable of binding and said monoclonal antibody, wherein a reaction between a bead-bound peptide and the fluorescently labeled monoclonal antibody causes an increase in the value of a fluorescent signal (F_m) emitted from said bead;

(d) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(e) identifying the subset to which each bead belongs and therefore the peptide on said bead as a function of the unique profile of classification parameter values; and

(f) detecting the particular epitope to which the monoclonal antibody binds as a function of the identification in step (e) and a change in the F_m values of the beads in each of said subsets in the sample relative to the F_m values of beads not reacted with said monoclonal antibody.

41. The method of claim 40 where said peptides are from 2 -100 amino acids in length.

42. A method of flow cytometric assay for antibodies reactive with given pathogens of interest in a fluid sample comprising the steps of:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently different from beads in any other subset in at least one of said

classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to an antigen derived from one of said pathogens of interest :

(b) obtaining a fluorescently labeled immunoglobulin-reactive reagent;

(c) mixing, to produce a reacted bead sample, the beadset with the fluid sample and the fluorescently labeled immunoglobulin-reactive reagent under conditions that will allow binding reactions between the bead-bound antigen and antibody in said sample and the fluorescently labeled immunoglobulin-reactive reagent wherein a reaction between a bead-bound antigen, antibody in said fluid sample and the fluorescently labeled reagent causes an increase in the value of a fluorescent signal (F_m) emitted from said bead;

(d) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(e) identifying the subset to which each bead belongs and therefore the peptide on said bead as a function of the unique profile of classification parameter values; and

(f) detecting the particular epitope to which the monoclonal antibody binds as a function of the identification in step (e) and a change in the F_m values of the beads in each of said subsets in the sample relative to the F_m values of beads not reacted with said fluid sample.

43. The method of claim 42 wherein said antigens comprise one or more of the following antigens: *Toxoplasma gondii*, Rubella virus, Cytomegalovirus, and Herpes Simplex virus.

44. The method of claim 43 wherein said fluorescently labeled immunoglobulin-reactive reagent is anti-Human IgG.

45. The method of claim 43 wherein said fluorescently labeled immunoglobulin-reactive reagent is anti-Human IgM

46. A method of flow cytometric assay for antibodies reactive with allergens of interest in a fluid sample comprising the steps of:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to an antigen derived from an allergen of interest;

(b) obtaining a fluorescently labeled IgE reactive reagent;

(c) mixing, to produce a reacted bead sample, the beadset with the fluid sample and the fluorescently labeled reagent under conditions that will allow binding

reactions between the bead-bound allergen and antibody in said sample and the fluorescently labeled IgE reactive reagent wherein a reaction between a bead-bound allergen, antibody in said fluid sample and the fluorescently labeled IgE-reactive reagent causes an increase in the value of a fluorescent signal (F_m) emitted from said bead;

(d) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(e) identifying the subset to which each bead belongs and therefore the allergen on said bead as a function of the unique profile of classification parameter values; and

(f) detecting the particular epitope to which the monoclonal antibody binds as a function of the identification in step (e) and a change in the F_m values of the beads in each of said subsets in the sample relative to the F_m values of beads not reacted with said fluid sample.

47. The method of claim 46 wherein said allergen comprise one or more of the following antigens: Junegrass, Red Top, Brome, Orchard, Timothy, Rye, Fesque, What, Quack, Bermuda, Johnson, Canary, Velvet, Saltgrass, Bahia, and Vernal.

48. The method of claim 46 wherein said fluorescently labeled IgE reactive reagent is anti-human IgE

49. The method of claim 46 wherein said fluorescently labeled IgE reactive reagent is anti-canine IgE.

50. A method of flow cytometric analysis capable of quantitating the concentration of an analyte of interest in a fluid sample comprising:

(a) obtaining a beadset comprising a plurality of subsets of beads, wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1 , C_2, C_3, ...C_n) values and sufficiently different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to a reactant that will specifically react with the selected analyte of interest in the sample to be tested; and

wherein the beads in a plurality of said subsets are coupled to the same reactant but at concentrations which differ among said subsets;

(b) mixing, to produce a reacted bead sample, the beadset with the fluid sample to be tested under conditions that will allow reactions between the analyte of interest in the fluid sample and the reactants on the beads in said set, wherein a reaction between a reactant and an analyte of interest on a bead causes a change in the value of a fluorescent signal (F_m) emitted from said bead;

(c) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(d) identifying the subset to which each bead belongs and therefore the concentration of reactant with which the bead was coupled as a function of the unique profile of classification parameter values; and

(e) detecting the concentration of the analyte of interest in said sample as a function of the identification in step (d) and the F_m values of the beads in each of said subsets relative to the F_m values of a second set of the beads in each of said subsets, wherein said beads in said second set have not been reacted with said fluid sample but have been reacted with a known concentration of the analyte of interest.

51. A method of flow cytometric analysis capable of quantitating the concentration of an analyte of interest in a fluid sample comprising:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1 , C_2, C_3, ...C_n) values and sufficiently different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to a reactant that will specifically react with the analyte of interest in the sample to be tested; and

wherein the beads in a plurality of said subsets are coupled to the same reactant but at concentrations which differ among said subsets;

(b) mixing, to produce a reacted bead sample, the beadset with a fluorescently labeled competitive inhibitor of the reaction between the analyte of interest and the reactant on the beads and with the fluid sample under conditions that will allow reactions between the analyte of interest in the fluid sample and the reactants on the beads in said set, wherein a reaction between an analyte of interest and a reactant on a bead causes a decrease in the value of a fluorescent signal (F_m) emitted from said bead;

(c) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(d) identifying the subset to which each bead belongs and therefore the concentration of reactant with which the bead was coupled as a function of the unique profile of classification parameter values;

(e) assigning a bead subset value to each bead subset with correlates relatively with the concentration of analyte with which the bead subset was coupled;

(f) determining an inter-bead subset slope from a plot of mean F_m for each bead subset versus bead subset value to produce an inter-bead subset slope; and

(g) determining the concentration of the analyte of interest in the sample by interpolation of the slope determined in step (f) into a standard assay curve wherein the inter-bead subset slopes of beads incubated with known

concentrations of the analyte of interest are plotted against the log of the known concentration of the analyte of interest.

52. A method of generating a multiplexed standard assay curve for use in quantitating the concentration of an analyte of interest in a fluid sample comprising the steps of:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in said subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1 , C_2, C_3, ...C_n) values and sufficiently different from beads in any other subset in at least one of said classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique; and

(2) are coupled to a reactant that will specifically react with a selected analyte of interest in a fluid to be tested; and

(3) wherein the beads in a plurality of said subsets are coupled to the same reactant but at concentrations which differ among said subsets;

(b) mixing, to produce a reacted bead sample, the beadset with a fluorescently labeled competitive inhibitor of the analyte of interest and a known concentration of the analyte of interest under conditions that will allow reactions between the analyte of interest in the fluid and the reactants on the beads in said set, wherein a reaction between a reactant and an analyte of interest on a bead causes a decrease in the value of a fluorescent signal (F_m) emitted from said bead;

(c) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(d) identifying the subset to which each bead belongs and therefore the concentration of reactant with which the bead was coupled as a function of the unique profile of classification parameter values; and

(e) assigning a bead subset value to each bead subset with correlates relatively with the concentration of analyte with which the bead subset was coupled; and

(f) determining an inter-bead subset slope from a plot of mean F_m for each bead subset versus bead subset value; and

(g) repeating steps (a) - (f) at least one time but with a known concentration of analyte of interest that differs from said concentration of analyte of interest employed in any other step (b); and

(h) plotting to produce a standard curve the inter-bead subset slopes at each known concentration of analyte of interest against the log of each known concentration of analyte of interest.

53. A method for flow cytometric analysis to detect a plurality of nucleic acid analytes of interest in a single sample comprising:

(a) obtaining a beadset comprising a plurality of subsets of beads wherein the beads in each subset;

(1) are sufficiently homogeneous with respect to each of at least three selected classification parameter (C_1, C_2, C_3 ... C_n) values and sufficiently different from beads in any other subset in at least one of said

classification parameter values so that the profile of classification parameter values within each subset detectable by flow cytometry is unique,

(2) are coupled to a nucleic acid that will specifically hybridize with a selected nucleic acid analyte of interest in a fluid to be tested,

(3) are reactive with a fluorescently labeled nucleic acid probe which competes with said nucleic acid analyte for hybridization with said nucleic acid coupled to the bead;

(b) mixing, to produce a reacted bead sample, the beadset with the fluid to be tested under conditions that will allow hybridization between nucleic acid analytes of interest in the fluid and the nucleic acids coupled to the beads in said beadset and thereby to allow the nucleic acid analytes in said fluid to inhibit hybridization between the fluorescently labeled nucleic acids with the nucleic acids coupled to said beads, resulting in a decrease in a fluorescent signal F_m emitted from a bead with which a nucleic acid analyte of interest in the fluid has reacted;

(c) analyzing the reacted sample by flow cytometry to determine the classification parameter value profile and an F_m value of each bead analyzed;

(d) identifying the subset to which each bead belongs and therefore the reactant on the bead as a function of the unique profile of classification parameter values; and

(e) detecting the presence or absence of a particular analyte of interest in said sample as a function of the identification in step (d) and an increase in the F_m values of

the beads in each of said subsets in the reacted fluid sample from the F_m values of the beads in each of said subsets not reacted with said fluid.

54. The method of claim 3 wherein said analytes are enzymes, said reactants are fluorescent molecules which upon reaction with the enzyme lose fluorescence, said change in F_m results from alteration of said substrates attached to said beads.

55. The method of claim 3 wherein said analytes are enzymes, said reactants are non-fluorescent molecules which upon reaction with the enzyme become fluorescent, and said change in F_m results from alteration of said substrates attached to said beads.

56. The method of claim 3 wherein said analytes are convertases which produce active enzymes from inactive precursors, said reactants are inactive precursors that are converted to active enzyme which in turn are reactants of fluorescently labeled substrates for said newly activated enzymes, and said change in F_m results from cleavage of said substrates from said beads.

57. The method of claim 5 wherein said analytes are enzymes, said reactants are molecules attached to a bead which, upon reaction with the enzyme, become ligates for a fluorescently labeled ligand, and wherein said change in F_m results from reaction of the new ligate with the fluorescently labeled ligand.

58. The method of claim 3 wherein said analyte is a cofactor which produces an active enzyme from an inactive apo-enzymes, said reactant is a fluorescently labeled substrate for said activated enzyme, and said change in F_m results from cleavage of said substrate from said active enzyme.

59. A method of processing a plurality of data signals generated by a flow cytometer in real-time, each said data signal being associated with a specific cytometric target and encoding a forward light scatter value, a side light scatter value, a red fluorescence value, an orange fluorescence value, and a green fluorescence value, comprising:

(a) receiving a data signal;

(b) extracting from said data signal (1) a forward light scatter value, (2) a side light scatter value, (3) a red fluorescence value, (4) an orange fluorescence value, and (5) a green fluorescence value;

(c) classifying said cytometric target into one of a plurality of classes, referred to as an identified class, said classification being a function of said extracted (1) forward light scatter value, (2) side light scatter value, (3) red fluorescence value, and (4) orange fluorescence value;

(d) incrementing a class-count value associated with said identified class, said class-count value encoding the number of cytometric targets classified as belonging to said identified class;

(e) accumulating a green-fluorescence-sum value associated with said identified class, said green fluorescence sum-value encoding an arithmetic sum of said extracted green fluorescence value for all cytometric targets classified as belonging to said identified class;

(f) repeating the operations described in paragraphs (a) through (e) for subsequent data signals;

(g) generating, for each of said plurality of classes, one or more outcome-description signals encoding textual information correlated with the class-count value and with the green-fluorescence-sum value for said class; and

(h) displaying said textual information.

60. The method of claim 59 wherein said specific cytometric target is an appropriately labeled bead.

61. The method of claim 60 wherein each one of said plurality of classes is associated with one bead subset, said bead subset formed in accordance with claim 2.

62. The method of claim 59 wherein the operation of paragraph © is further comprised of performing a reasonableness test on said cytometric target's identified class, said reasonableness test being a function of one or more of said identified class' (1) forward light scatter value, (2) side light scatter value, (3) red fluorescence value, and (4) orange fluorescence value.

63. A method of processing a plurality of data signals generated by a flow cytometer, each said data signal being associated with a specific flow cytometric target and encoding a plurality of classification parameter values and one or more measurement parameter values, comprising:

(a) receiving a data signal;

(b) extracting said plurality of classification parameter values and said one or more measurement parameter values from said data signal;

(c) classifying said cytometric target into one of a plurality of classes, referred to as an identified class, said classification being a function of said plurality of extracted classification parameter values;

(d) incrementing a class-count value associated with said identified class;

(e) accumulating each of said one or more extracted measurement values into one or more respective accumulation-values for said identified class;

(f) repeating the operations described in paragraphs (a) through (e) for subsequent data signals;

(g) generating, for each of said plurality of classes, one or more outcome-description signals encoding information correlated with the class-count and with the one or more accumulation-sum values for said class; and

(h) displaying said textual information.

64. The method of claim 63 wherein said processing in performed in real-time.

65. The method of claim 64 wherein each one of said plurality of classes is associated with one bead subset, said bead subset formed in accordance with claim 2.

66. The method of claim 63 wherein said specific cytometric target is an appropriately labeled bead.

67. The method of claim 63 wherein said data signal encodes a plurality of classification parameter values selected from the group consisting of forward light scatter, side light scatter, red fluorescence, and orange fluorescence.

68. The method of claim 63 wherein said data signal encodes one or more measurement parameter values selected from the group consisting of orange fluorescence and green fluorescence.

69. The method of claim 63 wherein said one or more outcome-description signals encodes textual information.

70. The method of claim 63 wherein each of said one or more outcome-description signals is determined by either an OVER-UNDER test or a SHIFT test.

71. An machine readable assay database, stored in a storage device, for the processing of flow-cytometric measurement data comprising:

(a) an assay definition table, said assay definition table encoding (1) one or more measurement subset token identifiers, (2) for each subset token identifier, one or more baseline measurement parameter values, and (3) for each subset token identifier, an interpretation test-type token;

(b) a discriminant function table, said discriminant function table encoding a classification decision tree based on one or more classification measurement parameters, said one or more classification measurement parameters encoded in said flow-cytometric measurement data;

(c) an interpretation table, said interpretation table encoding textual assay outcome-description information; and

(d) a results table, said results table capable of encoding statistical accumulation of real-time flow-cytometric measurement data.

72. A method of processing a plurality of data signals, in real-time, generated by a diagnostic device, each of said plurality of data signals being associated with a specific diagnostic target and encoding a plurality of classification parameter values and one or more measurement parameter values, comprising:

(a) receiving a data signal;

(b) extracting said plurality of classification parameter values and said one or more measurement parameter values from said data signal;

(c) classifying said diagnostic target into one of a plurality of classes, referred to as an identified class, said classification being a function of said plurality of extracted classification parameter values;

(d) incrementing a class-count value associated with said identified class;

(e) accumulating each of said one or more extracted measurement values into one or more respective accumulation-values for said identified class;

(f) repeating the operations described in paragraphs (a) through (e) for subsequent data signals;

(g) generating, for each of said plurality of classes, one or more outcome-description signals encoding information correlated with the class-count and with the one or more accumulation-sum values for said class; and

(h) displaying said outcome-description signals.

73. The method of claim 72 wherein said diagnostic device is selected from the group consisting of a flow cytometer and a cell sorter.

74. A program storage device that is readable by a computer, said program storage device having encoded therein a program of instructions that includes instructions for executing the method steps of a specified one of claims 59, 63, 71, and 72.

75. A method for flow cytometric analysis to detect genetic mutations in a DNA comprising:

(a) obtaining beads coupled to an oligonucleotide molecule designed to hybridize with a selected PCR product of interest;

(b) mixing the beads with said PCR product under conditions that will allow hybridization between said PCR product and the oligonucleotide coupled to the beads and thereby to allow the PCR product to inhibit hybridization between a fluorescently labeled nucleic acid probe that is completely complementary to said oligonucleotide coupled to said beads;

(c) adding said fluorescent probe to the mixture;

(d) analyzing the reacted sample by flow cytometry to determine the fluorescence of each bead analyzed; and

(e) detecting the genetic mutation or absence thereof as a result of the degree of fluorescence on the beads.

76. A method to detect a genetic mutations in a DNA comprising:

(a) obtaining beads coupled to an oligonucleotide molecule, said oligonucleotide molecule designed to hybridize with a selected PCR product of interest;

(b) mixing said beads with said PCR product, under conditions that will allow hybridization between said PCR product and the oligonucleotide coupled to the beads, to form a reacted mixture;

(c) adding a fluorescent probe to said reacted mixture;

(d) determining the fluorescence of the beads by flow cytometry; and

(e) detecting the genetic mutation, or absence thereof, as a result of the degree of the determined fluorescence on the beads.

77. A method of detecting a genetic mutation in a DNA comprising the steps of:

(a) selecting an oligonucleotide probe for said genetic mutation;

(b) preparing a fluorescent DNA probe complementary to the oligonucleotide probe coupling said selected probe to each one of a plurality of beads to form a bead aliquot;

(c) selecting PCR primers to amplify a region of said DNA corresponding to said selected probe;

(d) amplifying said genetic mutation by PCR to form PCR products;

(e) mixing said bead aliquot, said PCR products and said fluorescent probe to form a mixture;

(f) incubating said mixture to promote under competitive hybridization conditions;

(g) measuring the fluorescence said beads by flow cytometry; and

(h) detecting said genetic mutation, or absence thereof, as a function of the measured fluorescence of said beads.

78. The method of claim 77 wherein said genetic mutation is selected from the group consisting of mutations in MEN2a, MEN2b, MEN1, ret proto-oncogene, LDL receptor, NF1, NF type 2, BRCA1, BRCA2, BRCA3, APC, adenosine deaminase, XPAC, ERCC6 excision repair gene, fmr1, Duchenne's muscular dystrophy gene, myotonic dystrophy

protein kinase, androgen receptor, Huntington's, HPRT, apolipoprotein E, HEXA, steroid 2-hydroxylase, angiotensin, hNMLH1, 2 mismatch repair, APC, Rb, p53, bcr/abl, bcl-2 gene, chromosomes 11 to 14 and chromosomes 15 to 17 gene transpositions, and genes encoding ion transporters.

79. The method of claim 77 wherein said oligonucleotide probe has a length of between 5 and 500 nucleotides.

80. The method of claim 77 wherein said PCR primers are designed to amply a region of said DNA corresponding to said oligonucleotide probe.

81. The method of claim 77 wherein said fluorescent probe is selected from the group consisting of DNA sequences complementary to wild-type or mutant sequences coupled to the beads.

82. A kit for detection of a genetic mutation in a DNA comprising:

(a) a first container comprising beads coupled to an oligonucleotide designed to hybridize with a selected PCR product of interest;

(b) a second container comprising a PCR primer designed to amplify a section of DNA complementary to said oligonucleotide; and

(c) a third container comprising a fluorescent labeled DNA probe capable of selectively hybridizing said oligonucleotide.

83. The kit of claim 82, wherein said genetic mutation is selected from the group consisting of mutations in MEN2a, MEN2b, MEN1, ret proto-oncogene, LDL receptor, NF1, NF type 2, BRCA1, BRCA2, BRCA3, APC, adenosine deaminase, XPAC, ERCC6 excision

repair gene, fmr1, Duchenne's muscular dystrophy gene, myotonic dystrophy protein kinase, androgen receptor, Huntington's, HPRT, apolipoprotein E, HEXA, steroid 2-hydroxylase, angiotensin, hNMLH1, 2 mismatch repair, APC, Rb, p53, bcr/abl, bcl-2 gene, chromosomes 11 to 14 and chromosomes 15 to 17 gene transpositions, and genes encoding ion transporters.

84. The kit of claim 82 wherein said fluorescent labeled DNA probe has a length of between 5 and 500 nucleotides.

85. The method claim 3 where the analytes of interest are DNA segments, the reactant on the bead are DNA segment capable of specifically hybridizing to said analytes, and the fluorescent label is a fluorescent DNA segment also capable of specifically hybridizing with said reactant to compete with the hybridization of said reactant to said label.

Fig. 1

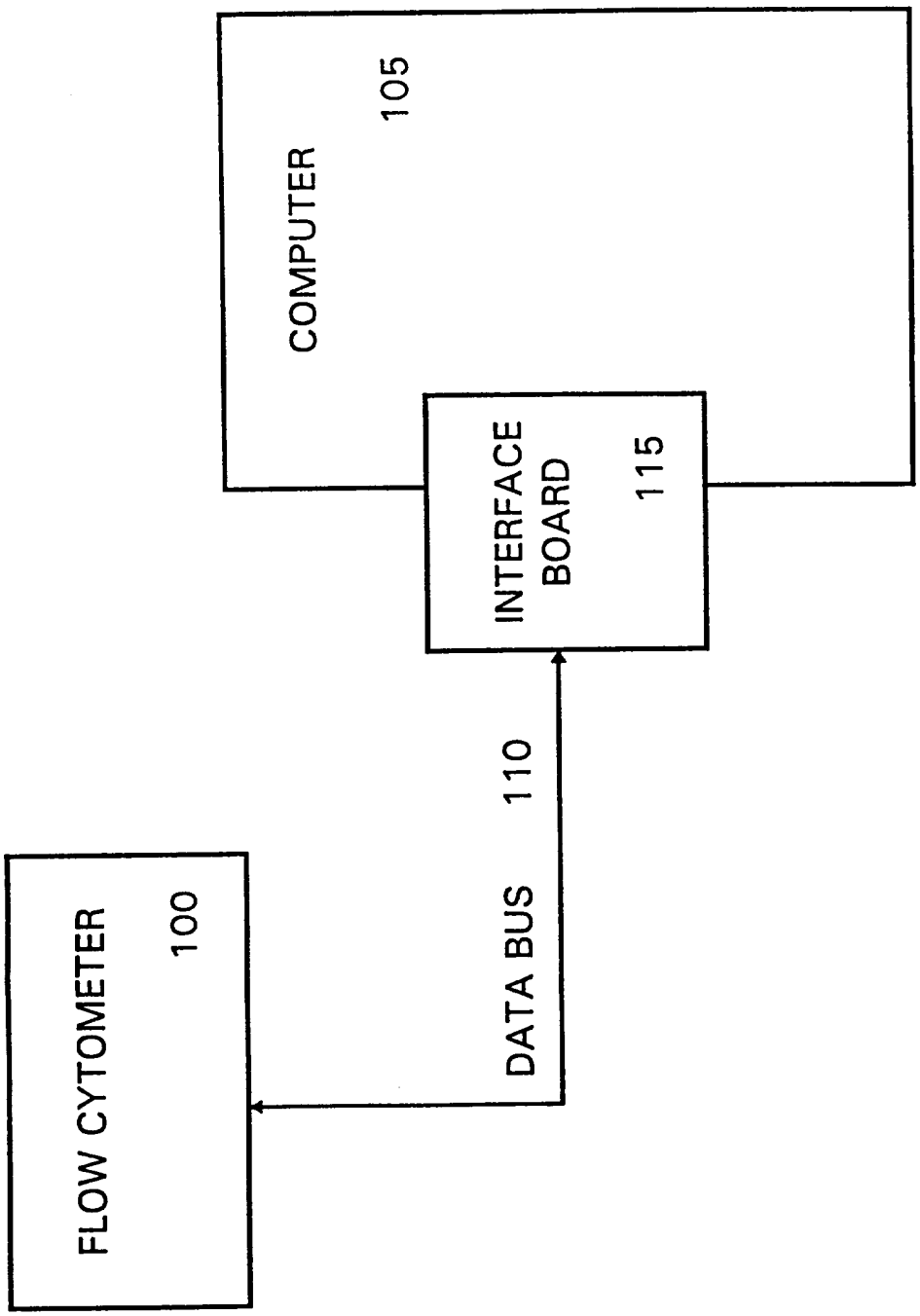

COMPUTER
105
INTERFACE BOARD
115
DATA BUS
110
FLOW CYTOMETER
100

Fig. 2

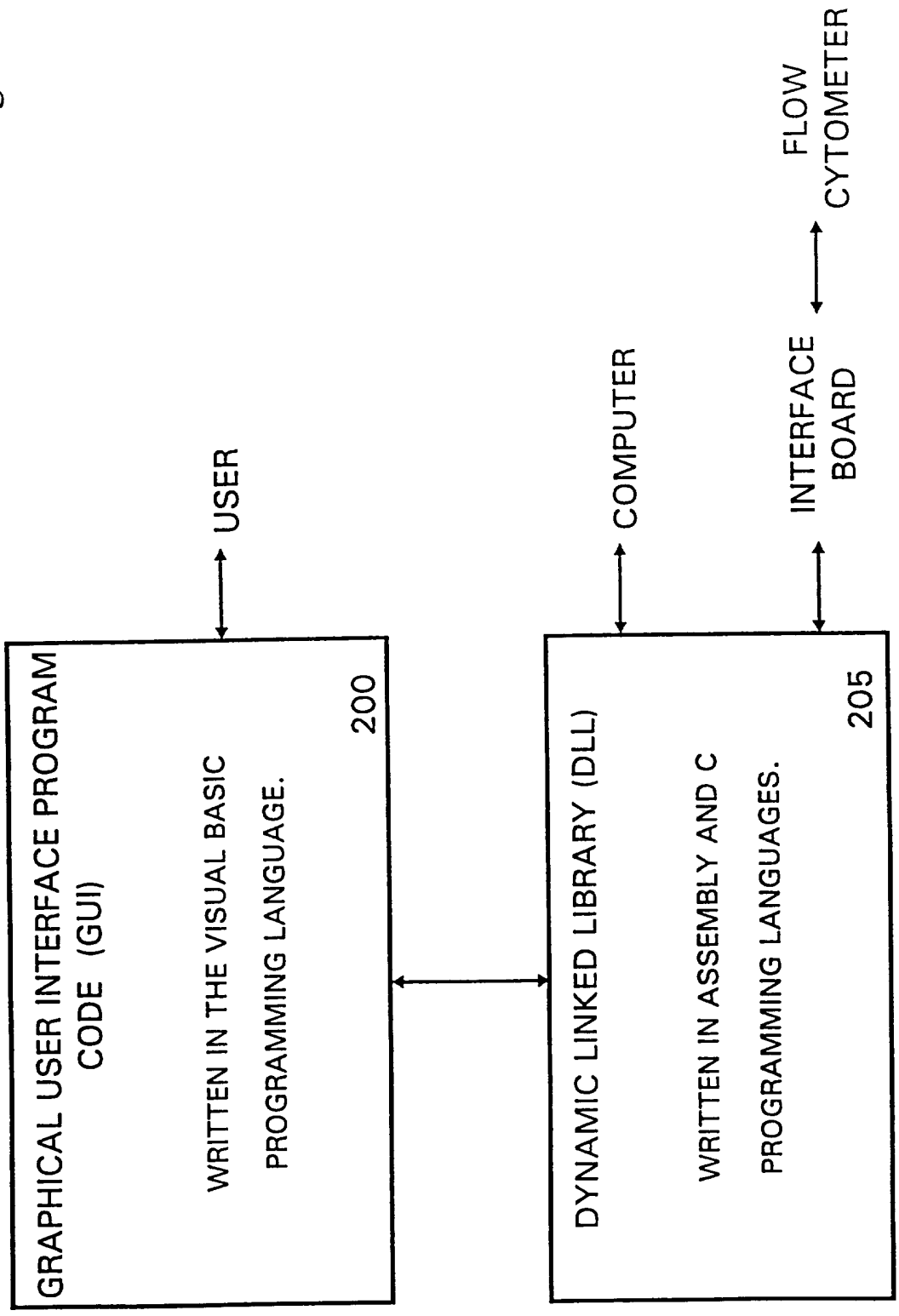

GRAPHICAL USER INTERFACE PROGRAM CODE (GUI)
WRITTEN IN THE VISUAL BASIC PROGRAMMING LANGUAGE.
200
USER
DYNAMIC LINKED LIBRARY (DLL)
WRITTEN IN ASSEMBLY AND C PROGRAMMING LANGUAGES.
205
COMPUTER
INTERFACE BOARD
FLOW CYTOMETER

Fig. 3

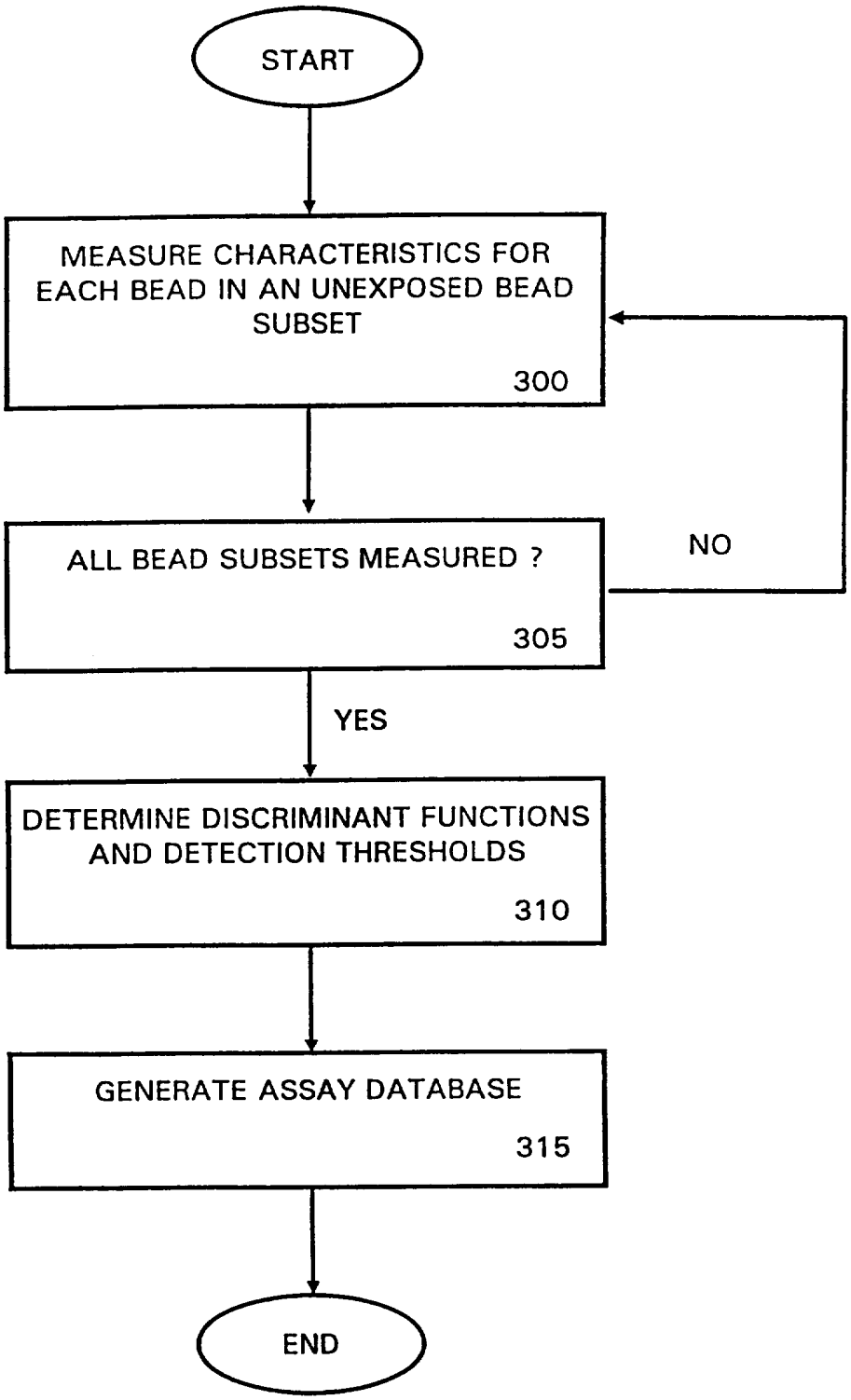

START
MEASURE CHARACTERISTICS FOR EACH BEAD IN AN UNEXPOSED BEAD SUBSET
300
ALL BEAD SUBSETS MEASURED ?
305
NO
YES
DETERMINE DISCRIMINANT FUNCTIONS AND DETECTION THRESHOLDS
310
GENERATE ASSAY DATABASE
315
END

Fig. 4

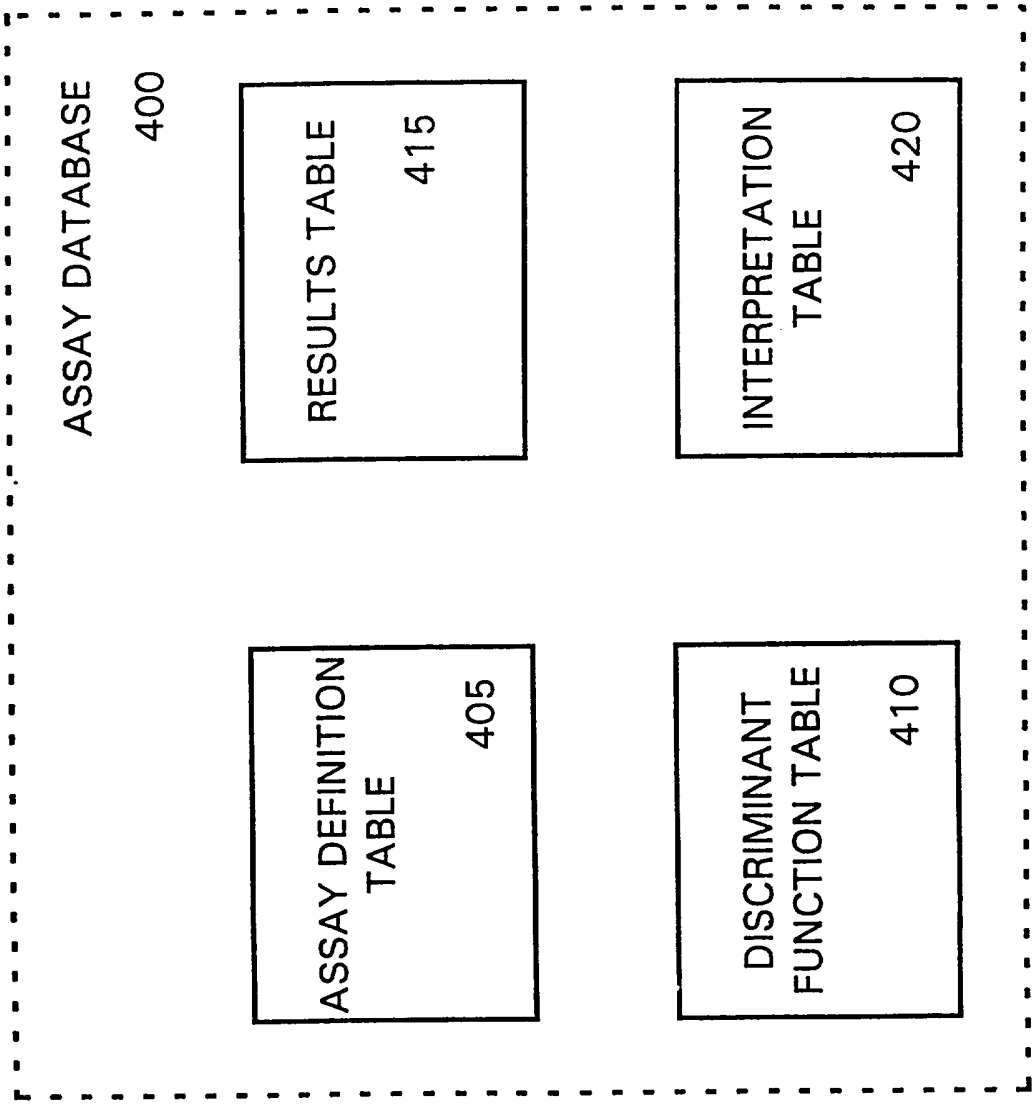

ASSAY DATABASE
400
RESULTS TABLE
415
INTERPRETATION TABLE
420
ASSAY DEFINITION TABLE
405
DISCRIMINANT FUNCTION TABLE
410

Fig. 5

SAMPLE BASELINE DATA ACQUISITION

ANALYTE	C_1: SIDE LIGHT SCATTER	C_2: FORWARD LIGHT SCATTER	C_3: ORANGE FLUORESCENCE	C_4: RED FLUORESCENCE	F_1: GREEN FLUORESCENCE
1	$\mu = 560$ $\sigma = 5.1$	*	$\mu = 0$	$\mu = 0$	$\mu = 170$ $\sigma = 1.3$
2	$\mu = 579$ $\sigma = 5.1$	*	$\mu = 48$ $\sigma = 0.69$	$\mu = 368$ $\sigma = 1.92$	$\mu = 170$ $\sigma = 1.3$
3	$\mu = 519$ $\sigma = 4.56$	*	$\mu = 98$ $\sigma = 0.99$	$\mu = 550$ $\sigma = 2.35$	$\mu = 170$ $\sigma = 1.3$
4	$\mu = 519$ $\sigma = 4.56$	*	$\mu = 0$	$\mu = 527$ $\sigma = 2.30$	$\mu = 170$ $\sigma = 1.3$

* Not used in illustrative example

Fig. 6

SAMPLE ASSAY DEFINITION TABLE

ASSAY NAME	SUBSET TOKEN	SUBSET NAME	F_1's BASE VALUE	F_1's STANDARD DEVIATION	TEST TYPE TOKEN
test_assay	46	KRAS CODON 46 WILDTYPE	170	1.3	0
test_assay	21	KRAS CODON 21 MUTANT	170	1.3	0
test_assay	50	KRAS CODON 50 MUTANT	170	1.3	0
test_assay	5	KRAS CODON 5 MUTANT	170	1.3	0

Fig. 7

SAMPLE DISCRIMINANT FUNCTION TABLE

ASSAY NAME	ROW ID	PARAMETER	LOW VALUE	HIGH VALUE	TRUE ROW ID	FALSE ROW ID	TRUE TOKEN	FALSE TOKEN
test_assay	0	C_1	500	620	1	0	0	0
test_assay	1	C_4	0	514	2	3	0	0
test_assay	2	C_4	0	200	0	0	50	5
test_assay	3	C_3	0	98	0	0	46	21

Fig. 8

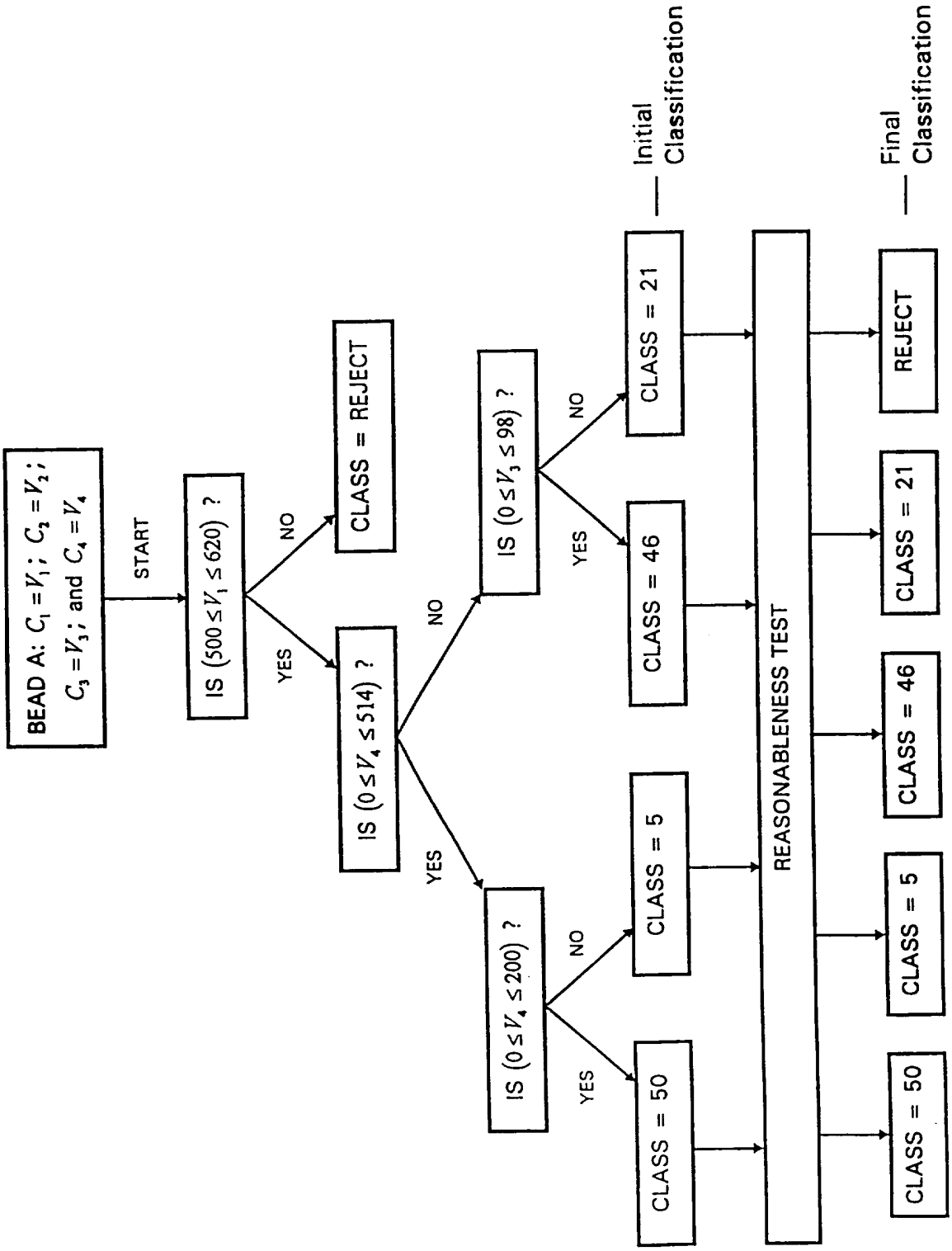

BEAD A: $C_1 = V_1$; $C_2 = V_2$; $C_3 = V_3$; and $C_4 = V_4$
START
IS $(500 \leq V_1 \leq 620)$?
NO
CLASS = REJECT
YES
IS $(0 \leq V_4 \leq 514)$?
NO
IS $(0 \leq V_3 \leq 98)$?
NO
CLASS = 21
YES
CLASS = 46
YES
IS $(0 \leq V_4 \leq 200)$?
NO
CLASS = 5
YES
CLASS = 50
Initial Classification
REASONABLENESS TEST
Final Classification
REJECT
CLASS = 21
CLASS = 46
CLASS = 5
CLASS = 50

Fig. 9

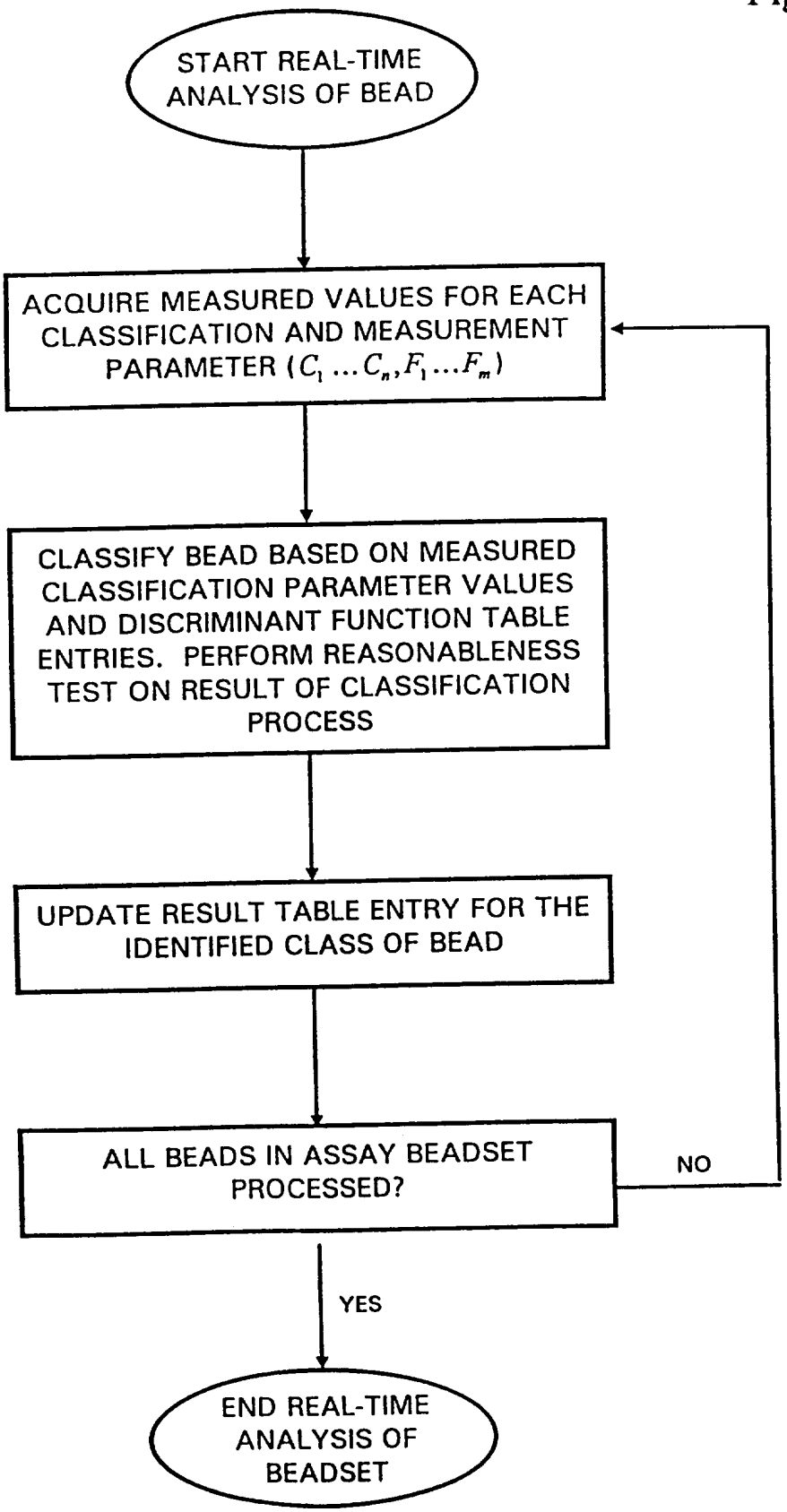

START REAL-TIME ANALYSIS OF BEAD
ACQUIRE MEASURED VALUES FOR EACH CLASSIFICATION AND MEASUREMENT PARAMETER ($C_1 \ldots C_n, F_1 \ldots F_m$)
CLASSIFY BEAD BASED ON MEASURED CLASSIFICATION PARAMETER VALUES AND DISCRIMINANT FUNCTION TABLE ENTRIES. PERFORM REASONABLENESS TEST ON RESULT OF CLASSIFICATION PROCESS
UPDATE RESULT TABLE ENTRY FOR THE IDENTIFIED CLASS OF BEAD
ALL BEADS IN ASSAY BEADSET PROCESSED?
NO
YES
END REAL-TIME ANALYSIS OF BEADSET

Fig. 10

SAMPLE RESULTS TABLE

ASSAY NAME	SUBSET TOKEN	COUNT	SUM	OVER COUNT	UNDER COUNT
test_assay	46	1,000	71,111	*	*
test_assay	21	1,000	90,000	*	*
test_assay	50	1,000	1,700,000	*	*
test_assay	5	1,000	70,000	*	*

* Not used in illustrative example

SAMPLE INTERPRETATION TABLE

Fig. 11

ASSAY NAME	SUBSET TOKEN	OUTCOME ID	TEST-TYPE TOKEN	LOW VALUE	HIGH VALUE	INTERPRETATION
test_assay	5	1	1	10	667	Identical complementary strand
test_assay	5	2	1	668	970	Similar oligo
test_assay	5	3	1	971	2,000	Not found in sample
test_assay	21	1	1	10	667	Identical complementary strand
test_assay	21	2	1	668	970	Similar oligo
test_assay	21	3	1	971	2,000	Not found in sample
test_assay	50	1	1	10	667	Identical complementary strand
test_assay	50	2	1	668	970	Similar oligo
test_assay	50	3	1	971	2,000	Not found in sample
test_assay	46	1	1	10	667	Identical complementary strand
test_assay	46	2	1	558	970	Similar oligo
test_assay	46	3	1	971	2,000	Not found in sample

Fig. 12

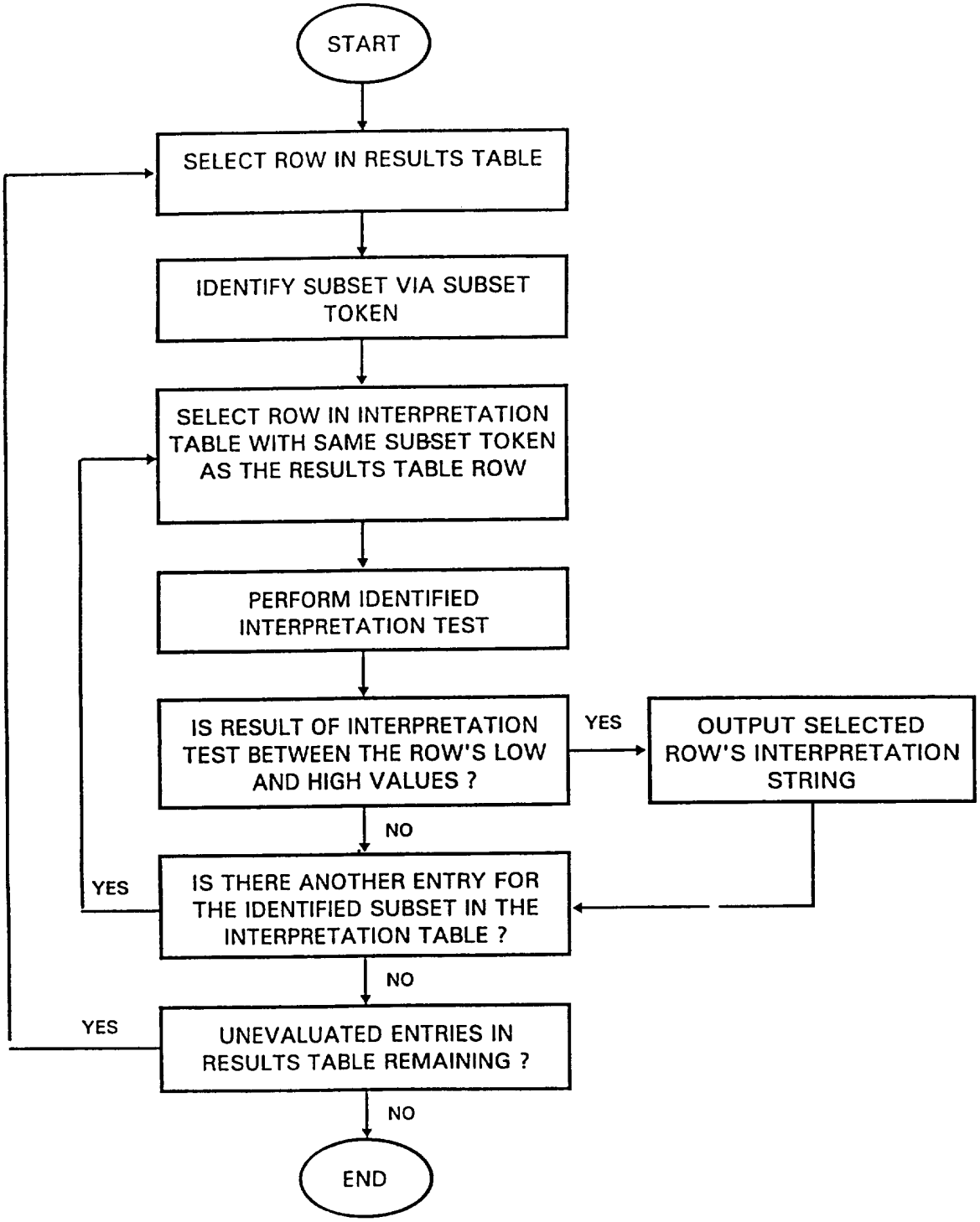

START
SELECT ROW IN RESULTS TABLE
IDENTIFY SUBSET VIA SUBSET TOKEN
SELECT ROW IN INTERPRETATION TABLE WITH SAME SUBSET TOKEN AS THE RESULTS TABLE ROW
PERFORM IDENTIFIED INTERPRETATION TEST
IS RESULT OF INTERPRETATION TEST BETWEEN THE ROW'S LOW AND HIGH VALUES ?
YES
OUTPUT SELECTED ROW'S INTERPRETATION STRING
NO
YES
IS THERE ANOTHER ENTRY FOR THE IDENTIFIED SUBSET IN THE INTERPRETATION TABLE ?
NO
YES
UNEVALUATED ENTRIES IN RESULTS TABLE REMAINING ?
NO
END

Fig. 13a

Assay Definition Table

Antigen	Microsphere identified via dye color	Antigen-Microsphere Complex	Fluorescent Antibody (F_m)	Subset Token
LH	1 μm Crimson Beads	LH—Crimson Beads	anti-LH	18
TSH	1 μm Dark Red Beads	TSH—Dark Red Beads	anti-TSH	45
IgA	3 μm Clear Beads	IgA — Clear Beads	anti-Ig	50

Fig. 13b

Baseline Data Table

Bead Subset dye color (token)	C_1 mean side light scatter	C_2 mean orange fluorescence	C_3 mean red fluorescence	F_m mean green fluorescence
Crimson (18)	38.46	1.71	30.69	9.15
Dark Red (45)	40.37	0.00	88.62	5.44
Clear (50)	167	0.00	0.00	7.83

Fig. 13c

Discriminant Function Table

Row ID	Parameter	Low Value	High Value	True Node	False Node	True Token	False Token
0	C_3	0	5	3	1	0	0
1	C_1	400	420	2	0	0	0
2	C_3	0	454	0	0	18	45
3	C_1	560	580	0	0	50	0

Fig. 13d

Interpretation Table

— $F_m \pm 1.5$ log(baeline F_m value) —

Subset Token	Outcome ID	Test-Type Token	Low Value	High Value	Interpretation
18	1	1	289.35	-	Anti-LH found.
18	2	1	-	289.35	Anti-LH not found.
45	1	1	172.03	-	Anti-TSH found.
45	2	1	-	172.03	Anti-TSH not found.
50	1	1	247.61	-	Anti-IgA found.
50	2	1	-	247.61	Anti-IgA not found.

Fig. 13e

Results Table

Sample	Antibody Present	Measured F_m Subset 18	Measured F_m Subset 45	Measured F_m Subset 50
1	Anti—IgA	70	10	6049
2	anti—LH	132	442	180
3	anti—TSH	2124	108	182
4	anti—IgA + anti—TSH	2152	115	5917

Fig. 14

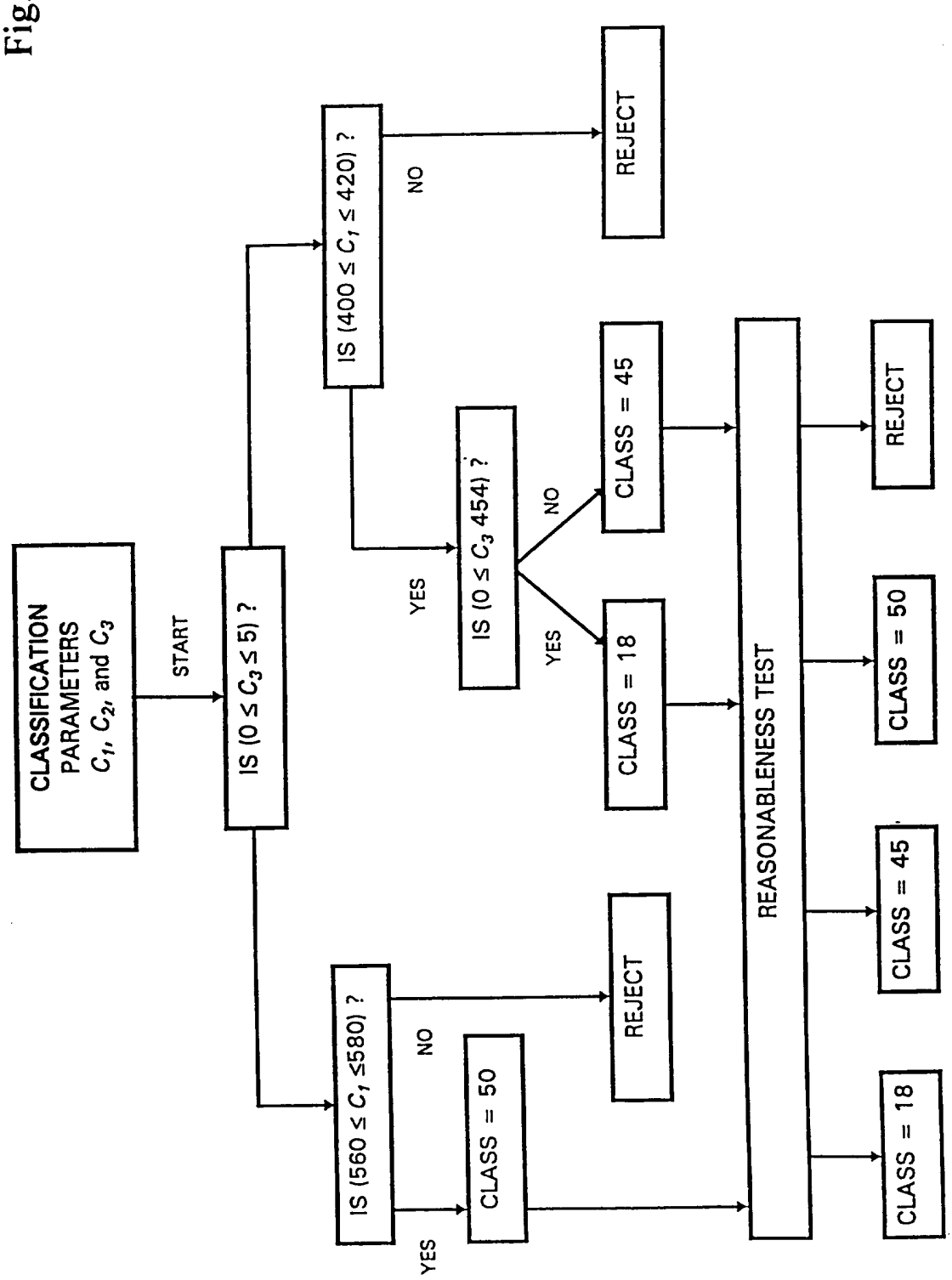

CLASSIFICATION PARAMETERS C_1, C_2, and C_3
START
IS $(0 \leq C_3 \leq 5)$?
IS $(400 \leq C_1 \leq 420)$?
NO
REJECT
YES
IS $(0 \leq C_3 454)$?
NO
CLASS = 45
YES
CLASS = 18
IS $(560 \leq C_1 \leq 580)$?
NO
REJECT
YES
CLASS = 50
REASONABLENESS TEST
REJECT
CLASS = 50
CLASS = 45
CLASS = 18

Fig. 15a

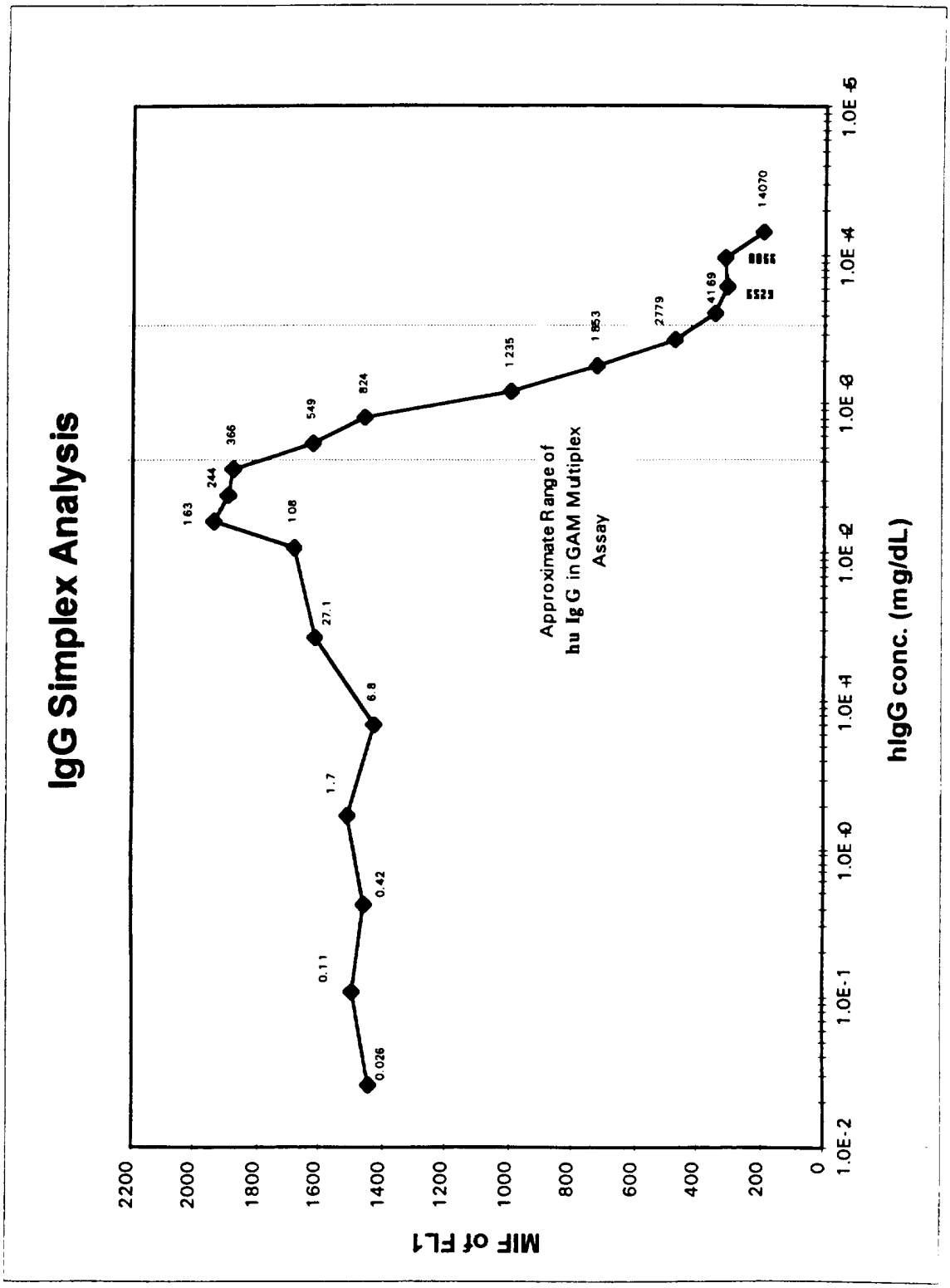
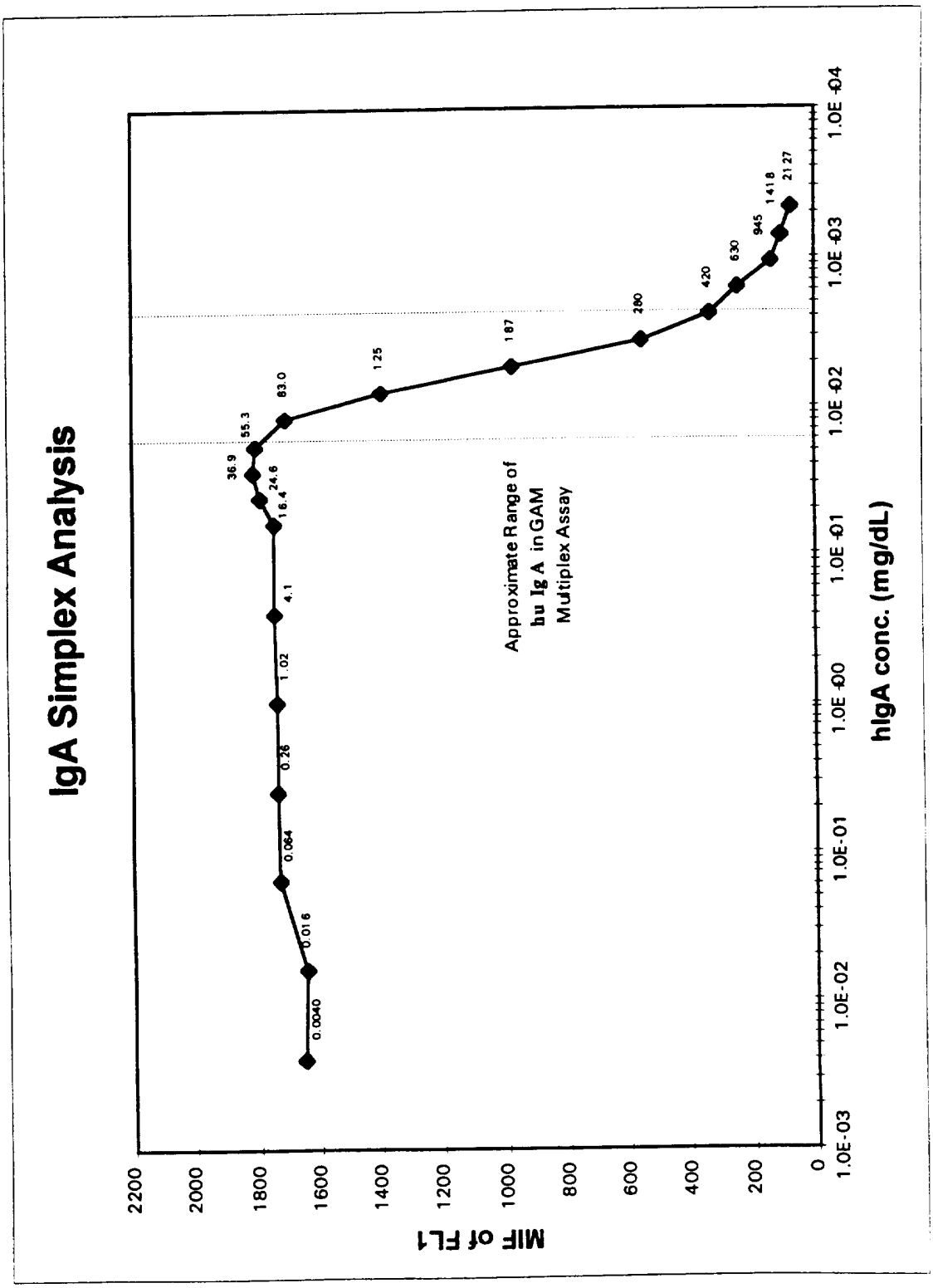

IgG Simplex Analysis
MIF of FL1
hIgG conc. (mg/dL)
Approximate Range of hu IgG in GAM Multiplex Assay
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1.0E-2
1.0E-1
1.0E-0
1.0E+1
1.0E-2
1.0E-3
1.0E+4
1.0E-5
0.026
0.11
0.42
1.7
6.8
27.1
108
163
244
366
549
824
1235
1853
2779
4169
5523
8865
14070

Fig. 15b


IgA Simplex Analysis
MIF of FL1
hIgA conc. (mg/dL)
Approximate Range of
hu Ig A in GAM
Mutiplex Assay
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1.0E-03
1.0E-02
1.0E-01
1.0E-00
1.0E-01
1.0E-02
1.0E-03
1.0E-04
0.0040
0.016
0.064
0.26
1.02
4.1
16.4
24.6
36.9
55.3
83.0
125
187
280
420
630
945
1418
2127

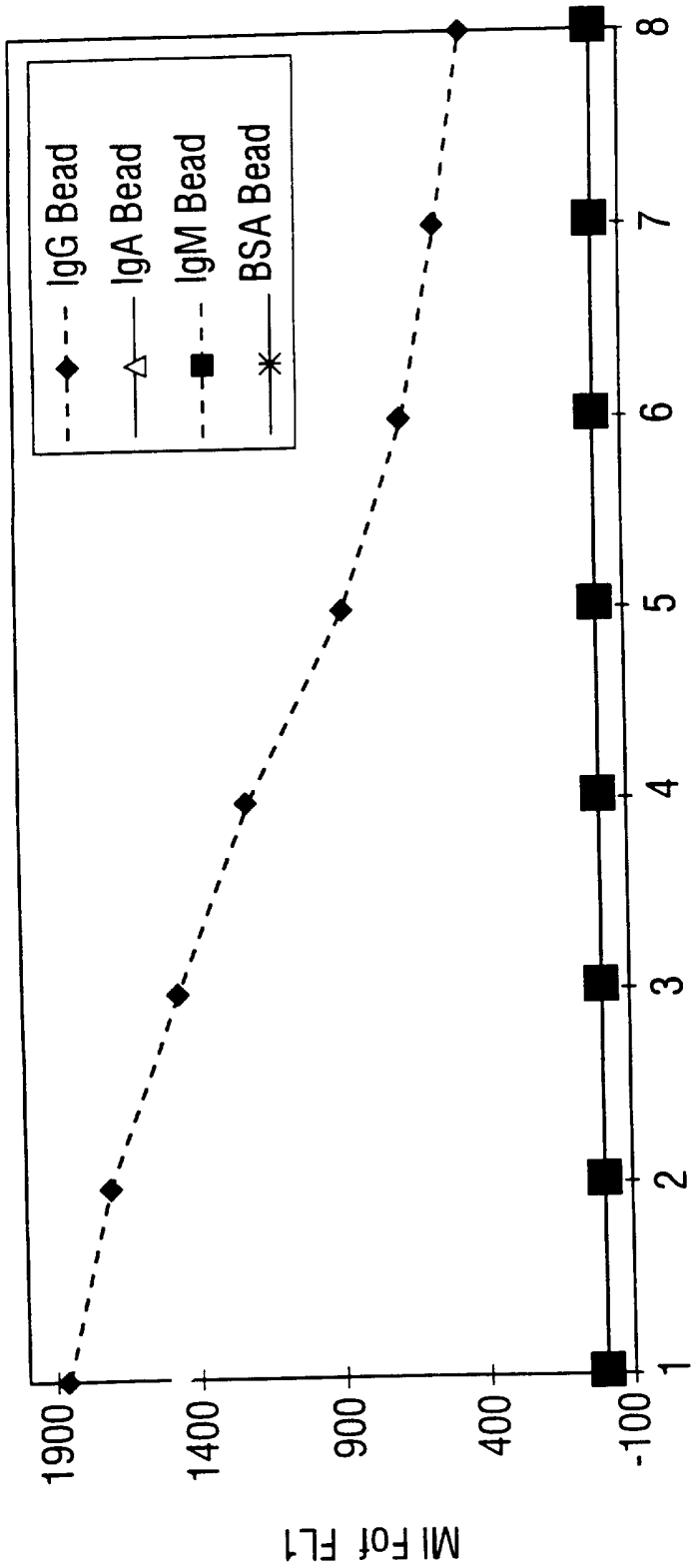

IgG Bead
IgA Bead
IgM Bead
BSA Bead
1900
1400
900
400
-100
1
2
3
4
5
6
7
8
MI For FL1
Tube No.

FIG. 16A

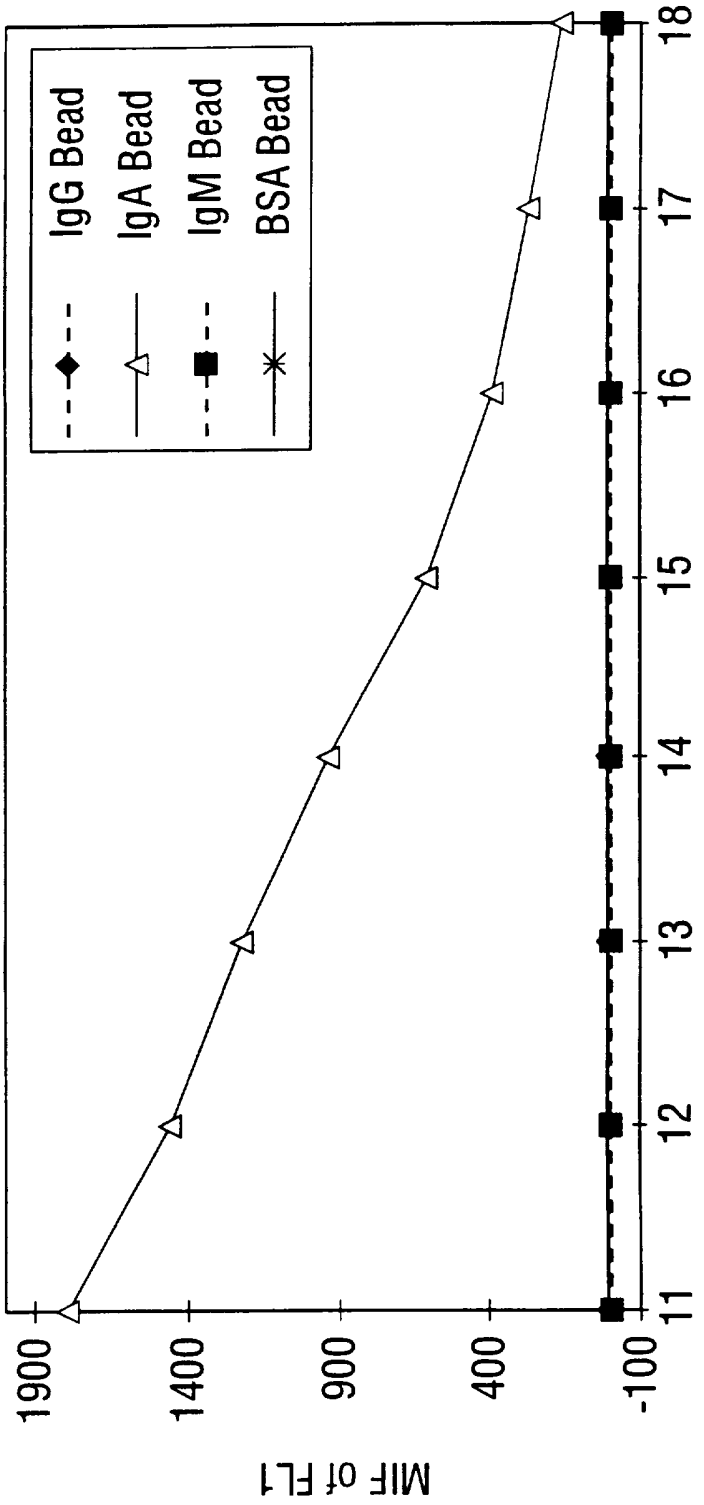

IgG Bead
IgA Bead
IgM Bead
BSA Bead
MIF of FL1
1900
1400
900
400
-100
11
12
13
14
15
16
17
18
Tube No.

FIG. 16B

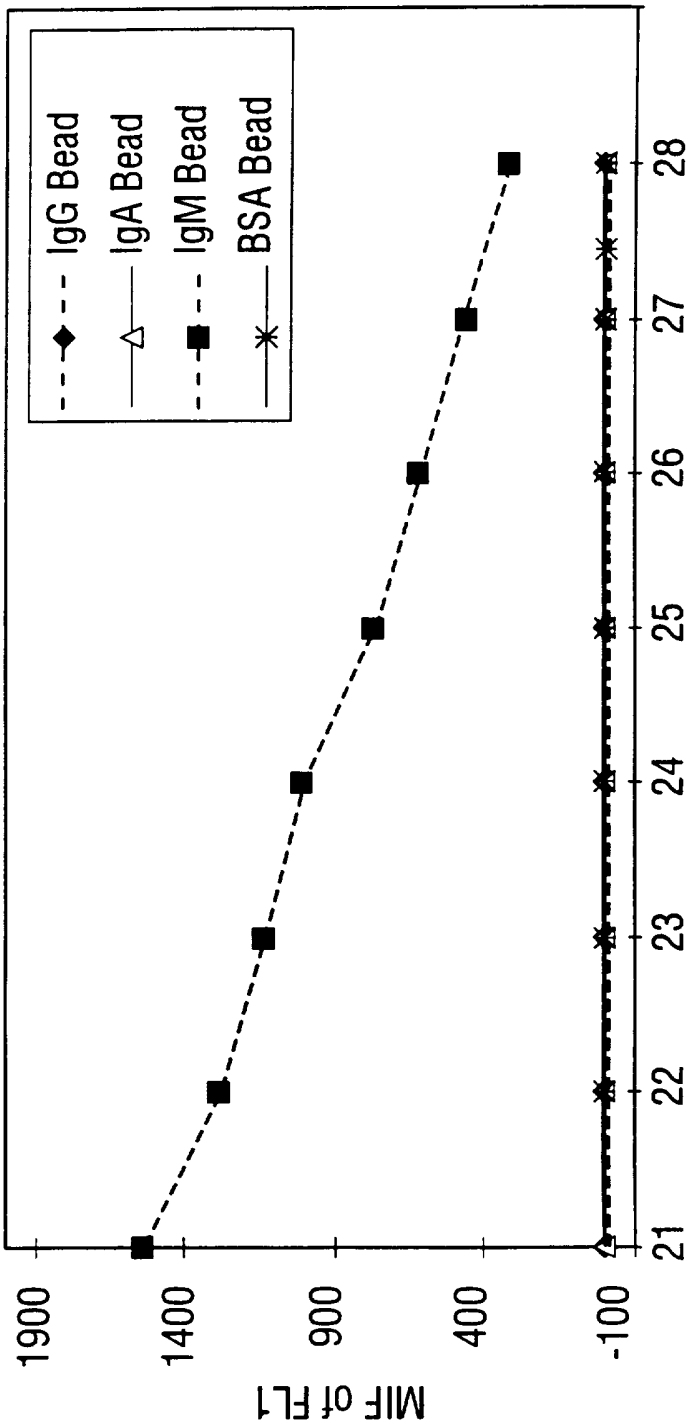

IgG Bead
IgA Bead
IgM Bead
BSA Bead
MIF of FL1
1900
1400
900
400
-100
21
22
23
24
25
26
27
28
Tube No.

FIG. 16C

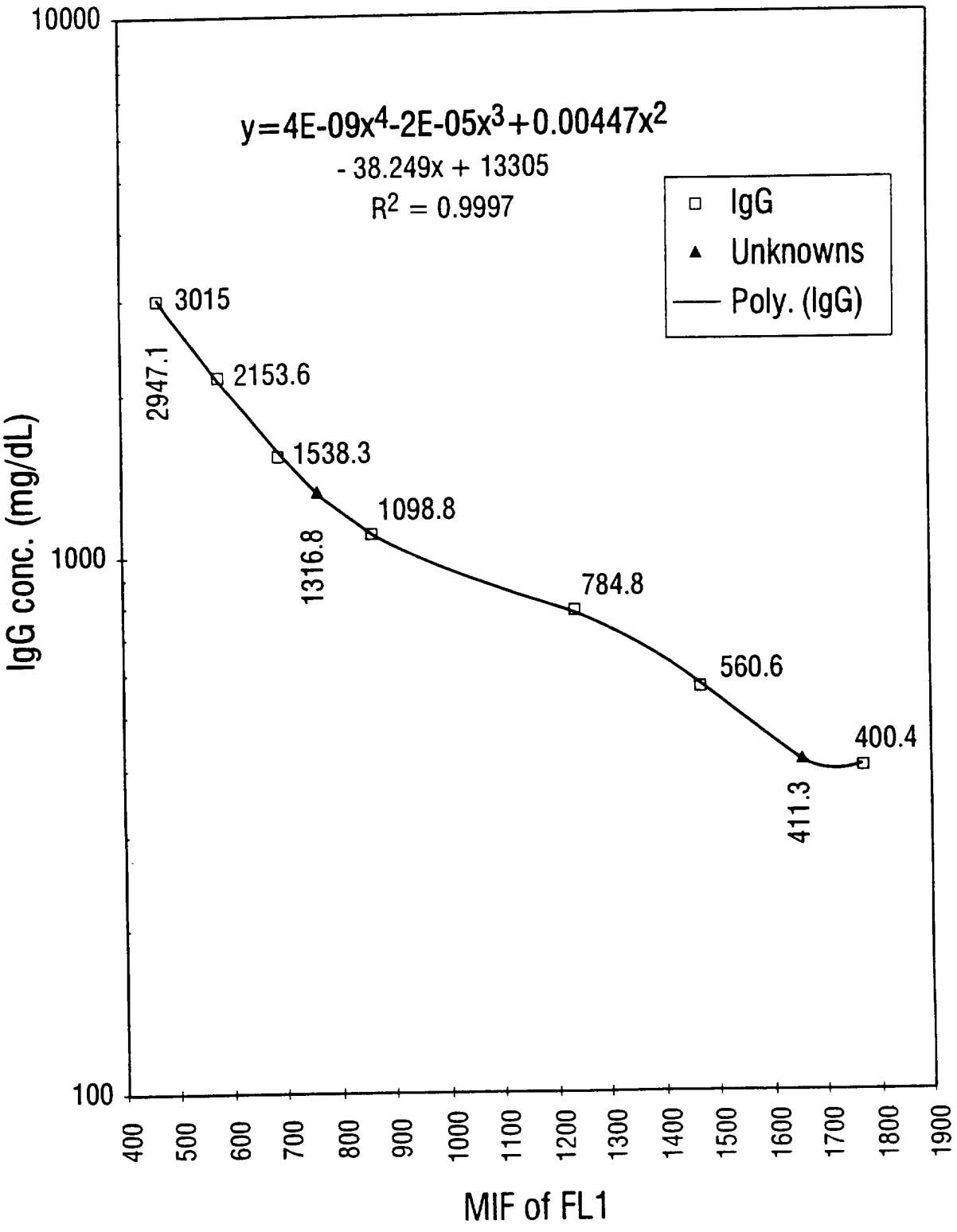

10000
1000
100
IgG conc. (mg/dL)
y=4E-09x4-2E-05x3+0.00447x2
- 38.249x + 13305
R2 = 0.9997
IgG
Unknowns
Poly. (IgG)
3015
2947.1
2153.6
1538.3
1316.8
1098.8
784.8
560.6
411.3
400.4
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
MIF of FL1

FIG. 17

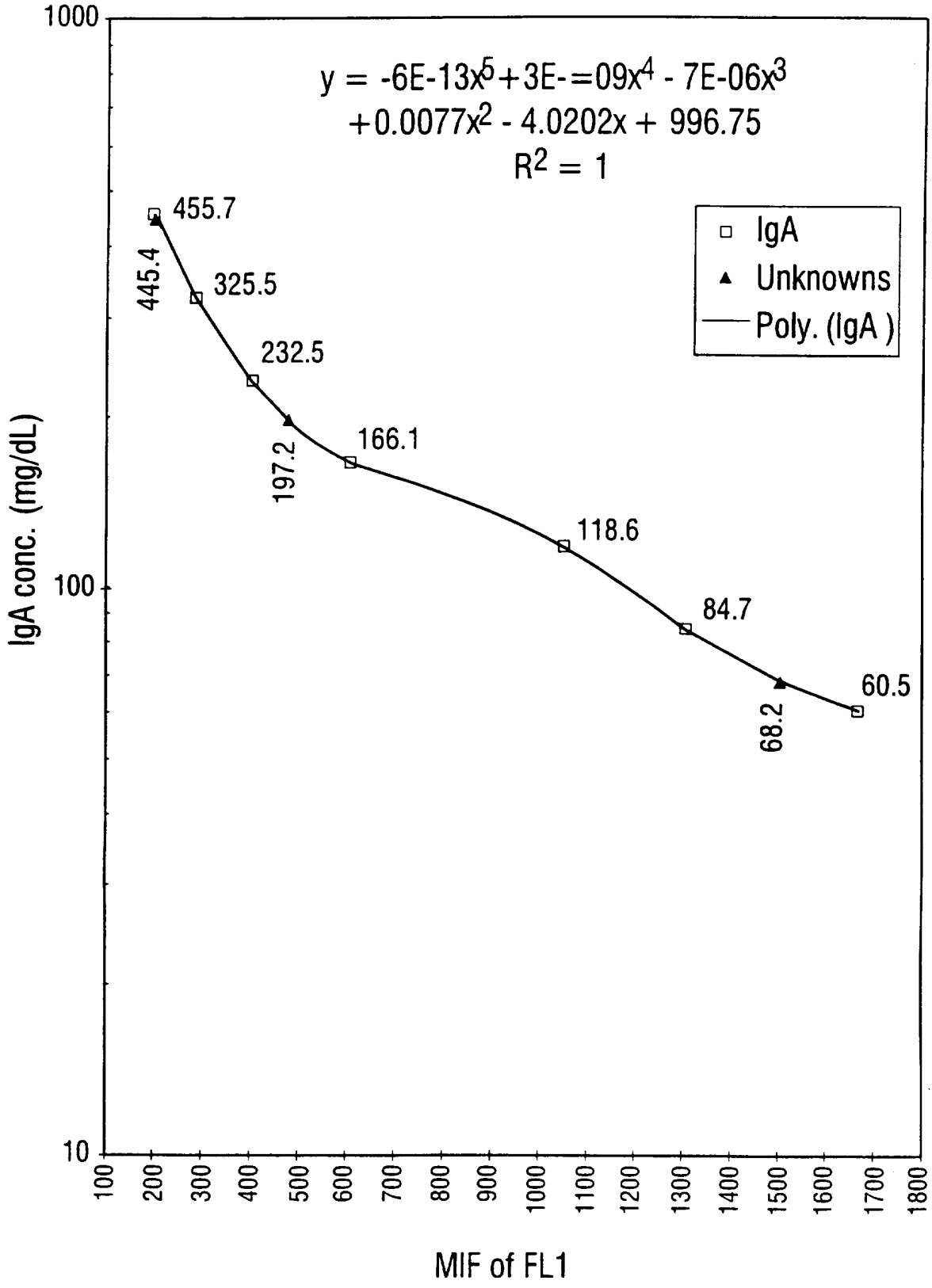

y = -6E-13x5+3E-=09x4 - 7E-06x3
+0.0077x2 - 4.0202x + 996.75
R2 = 1
IgA
Unknowns
Poly. (IgA)
455.7
445.4
325.5
232.5
197.2
166.1
118.6
84.7
68.2
60.5
IgA conc. (mg/dL)
1000
100
10
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
MIF of FL1

FIG. 18

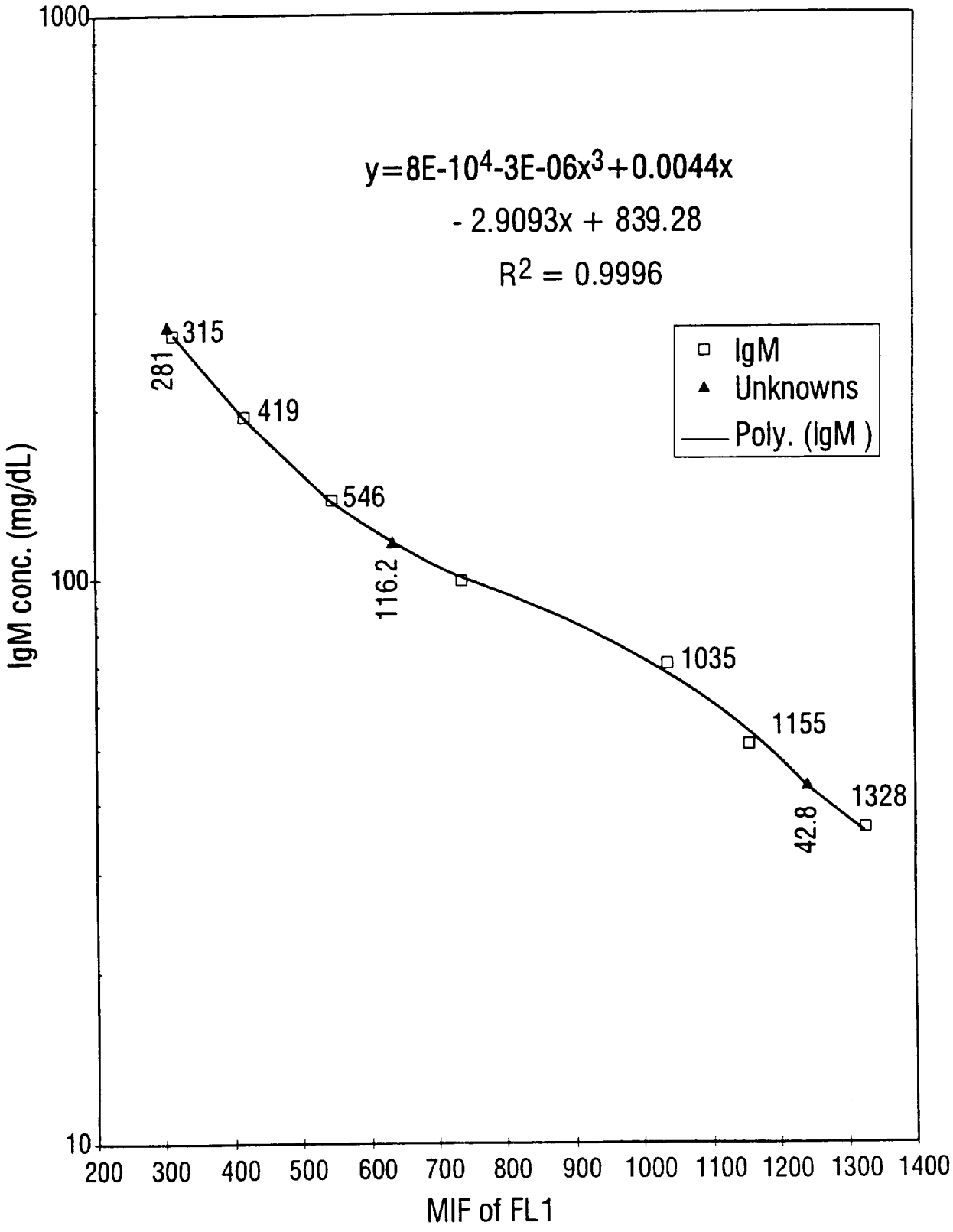

1000
100
10
y=8E-10^4-3E-06x^3+0.0044x
- 2.9093x + 839.28
R^2 = 0.9996
IgM
Unknowns
Poly. (IgM)
IgM conc. (mg/dL)
315
281
419
546
116.2
1035
1155
1328
42.8
200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400
MIF of FL1

FIG. 19

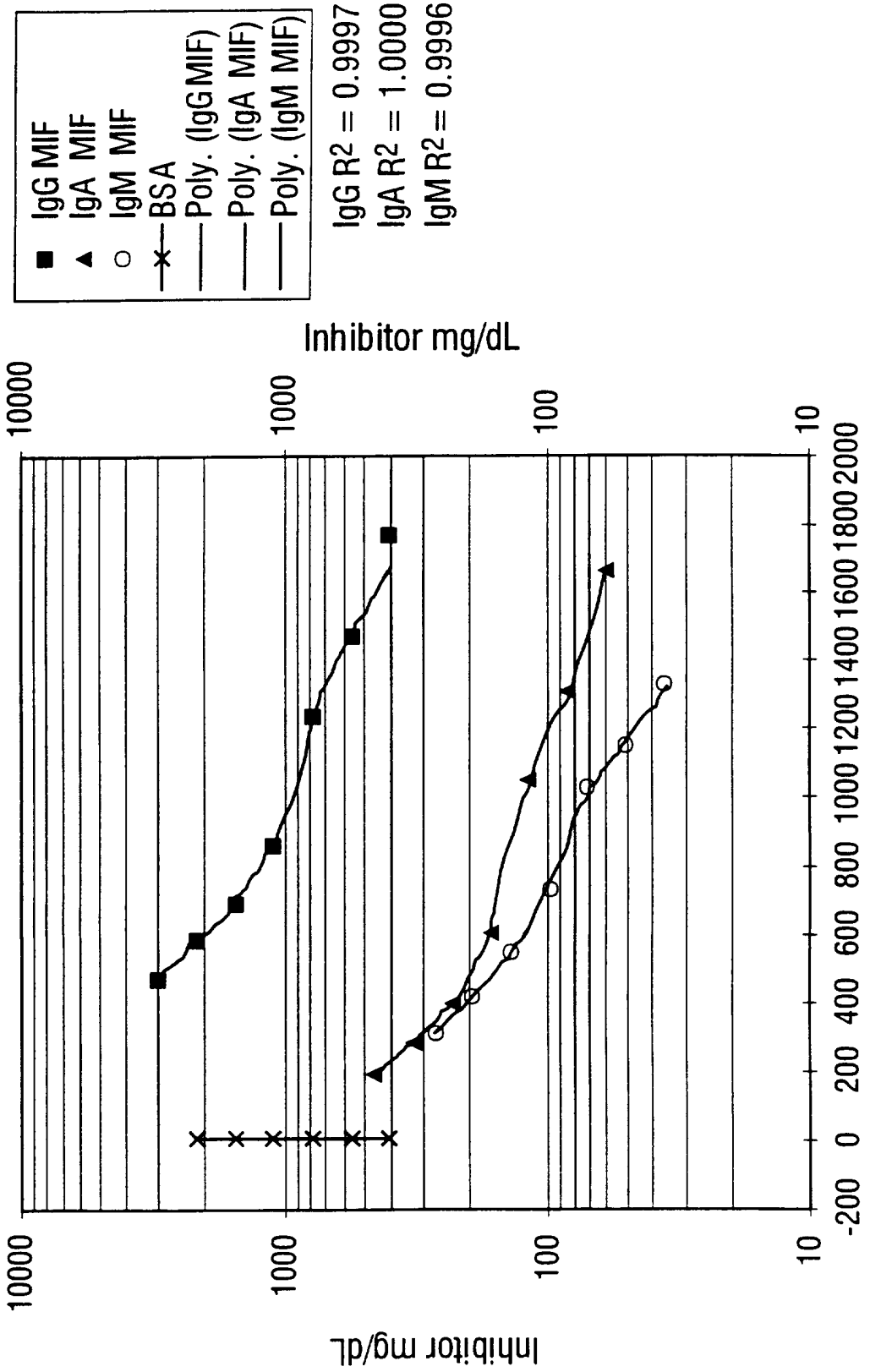

IgG MIF
IgA MIF
IgM MIF
BSA
Poly. (IgG MIF)
Poly. (IgA MIF)
Poly. (IgM MIF)
IgG R2 = 0.9997
IgA R2 = 1.0000
IgM R2 = 0.9996
Inhibitor mg/dL
10000
1000
100
10
-200 0 200 400 600 800 1000 1200 1400 1600 1800 2000
MIF of FL1

FIG. 20

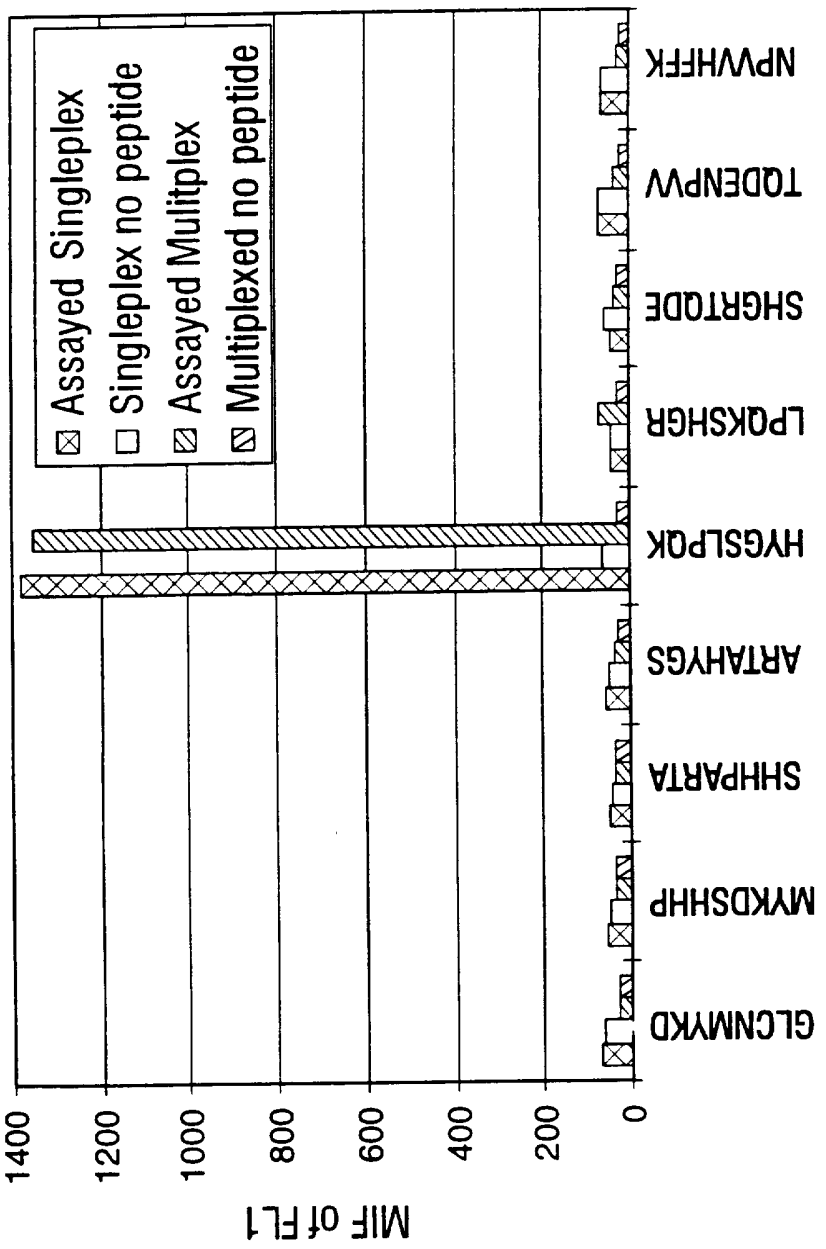

Assayed Singleplex
Singleplex no peptide
Assayed Mulitplex
Multiplexed no peptide
MIF of FL1
1400
1200
1000
800
600
400
200
0
GLCNMYKD
MYKDSHHP
SHHPARTA
ARTAHYGS
HYGSLPQK
LPQKSHGR
SHGRTQDE
TQDENPVV
NPVVHFFK
Peptides # 1-9

FIG. 21

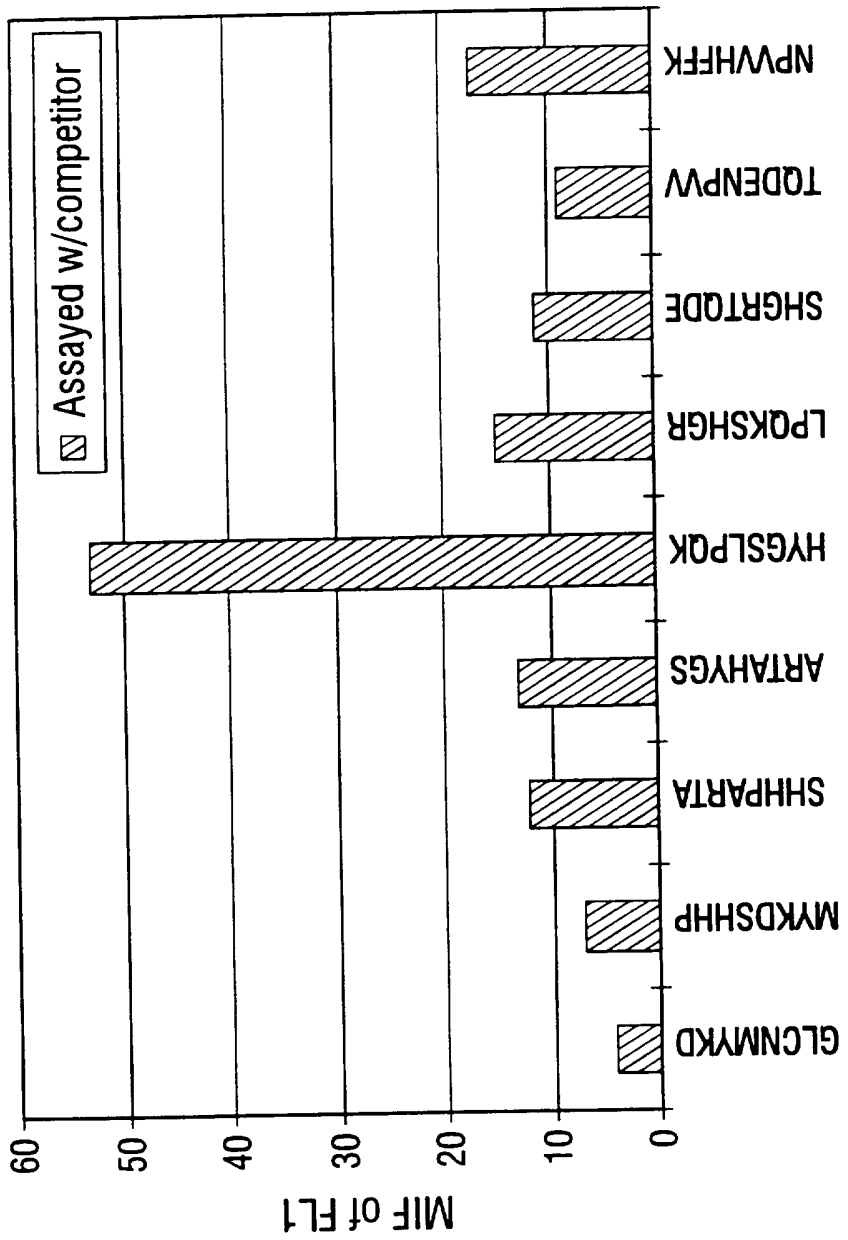

Assayed w/competitor
GLCNMVKD
MVKDSHHP
SHHPARTA
ARTAHVGS
HVGSLPQK
LPQKSHGR
SHGRTQDE
TQDENPVV
NPVVHFFK
0
10
20
30
40
50
60
MIF of FL1
Peptide

FIG. 22

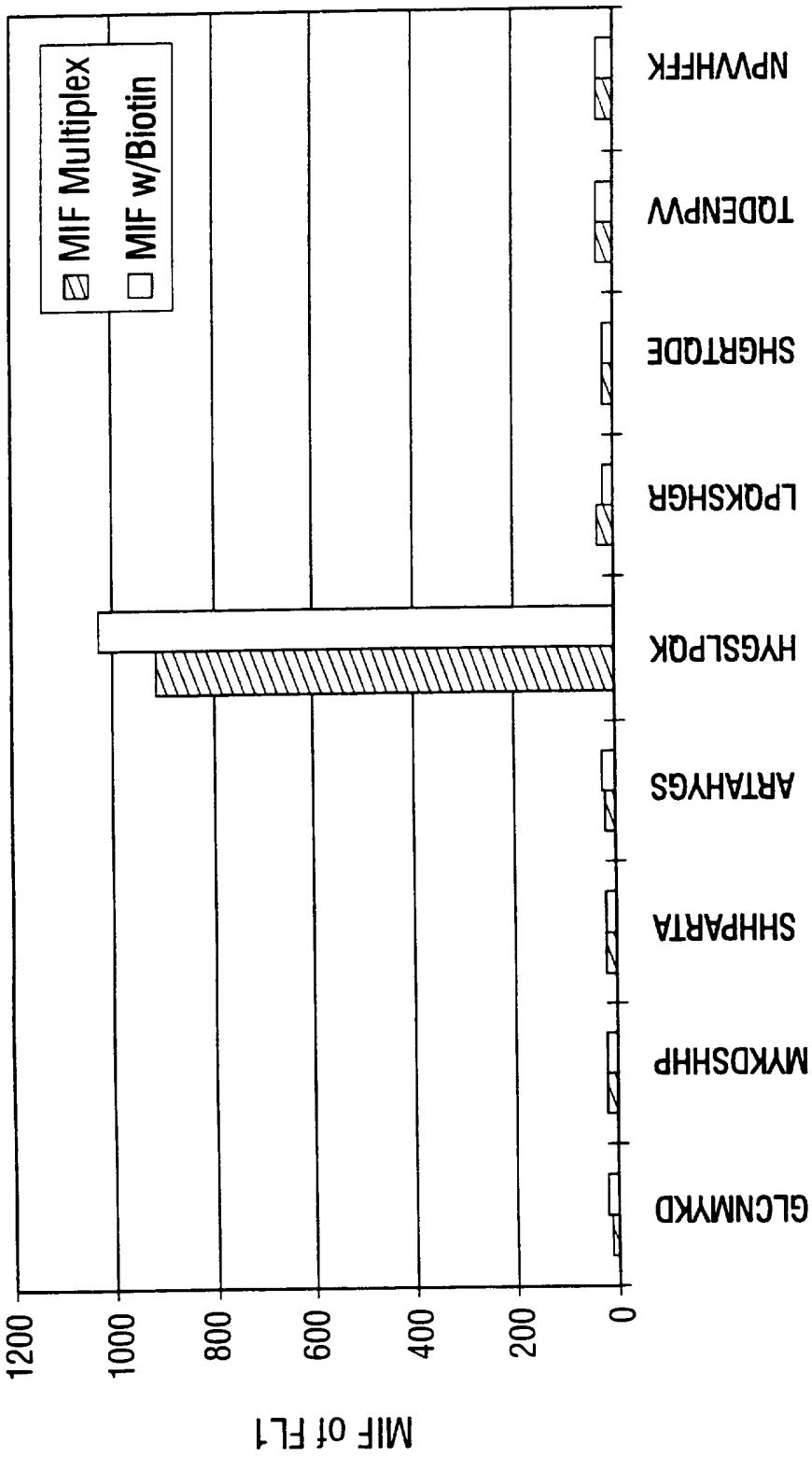

MIF Multiplex
MIF w/Biotin
MIF of FL1
1200
1000
800
600
400
200
0
GLCNMVKD
MYKDSHHP
SHHPARTA
ARTAHYGS
HYGSLPQK
LPQKSHGR
SHGRTQDE
TQDENPVV
NPVVHFFK
Peptide

FIG. 23

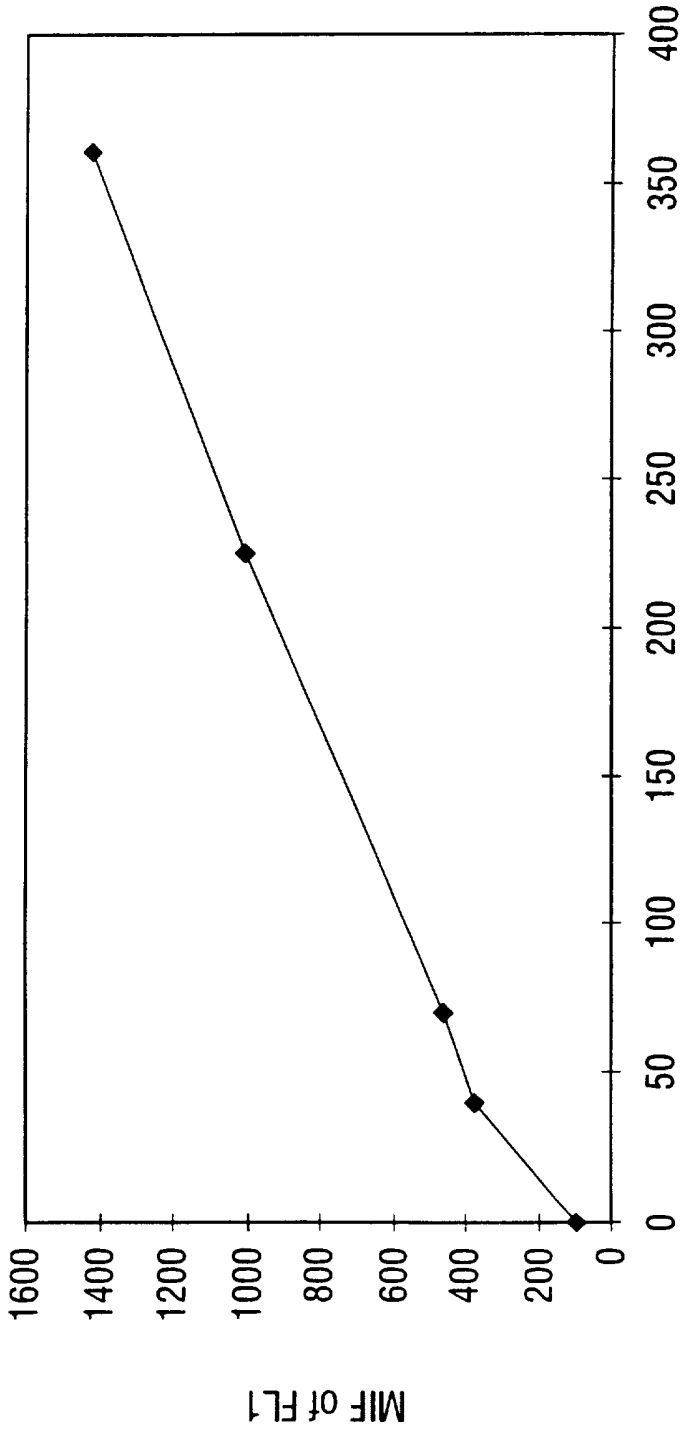

MIF of FL1
1600
1400
1200
1000
800
600
400
200
0
0
50
100
150
200
250
300
350
400
Anti Rubella IgG (IU/mL)

FIG. 24

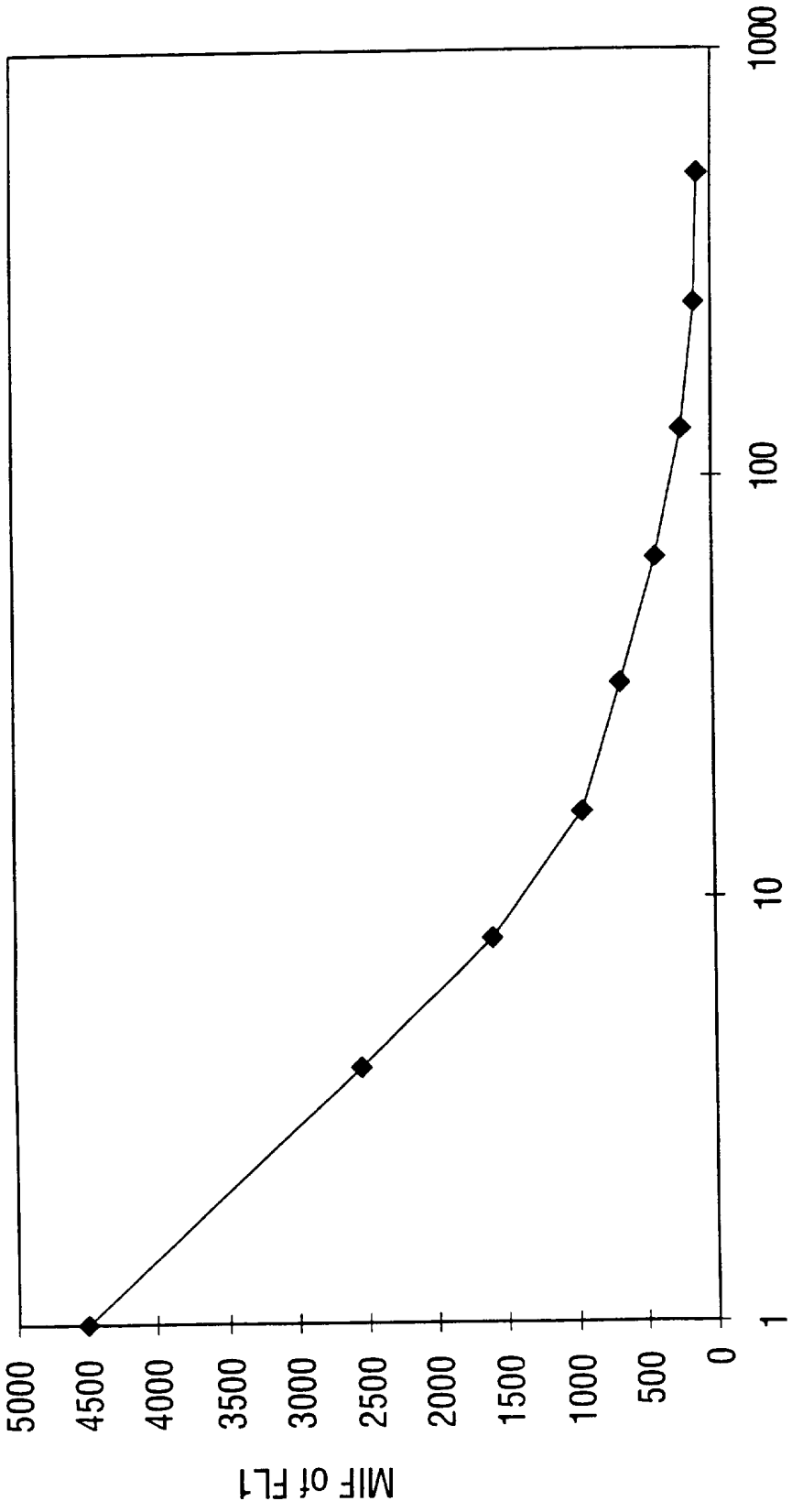

MIF of FL1
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1
10
100
1000
Reciprocal of Calibrator 70 Dilution

FIG. 25

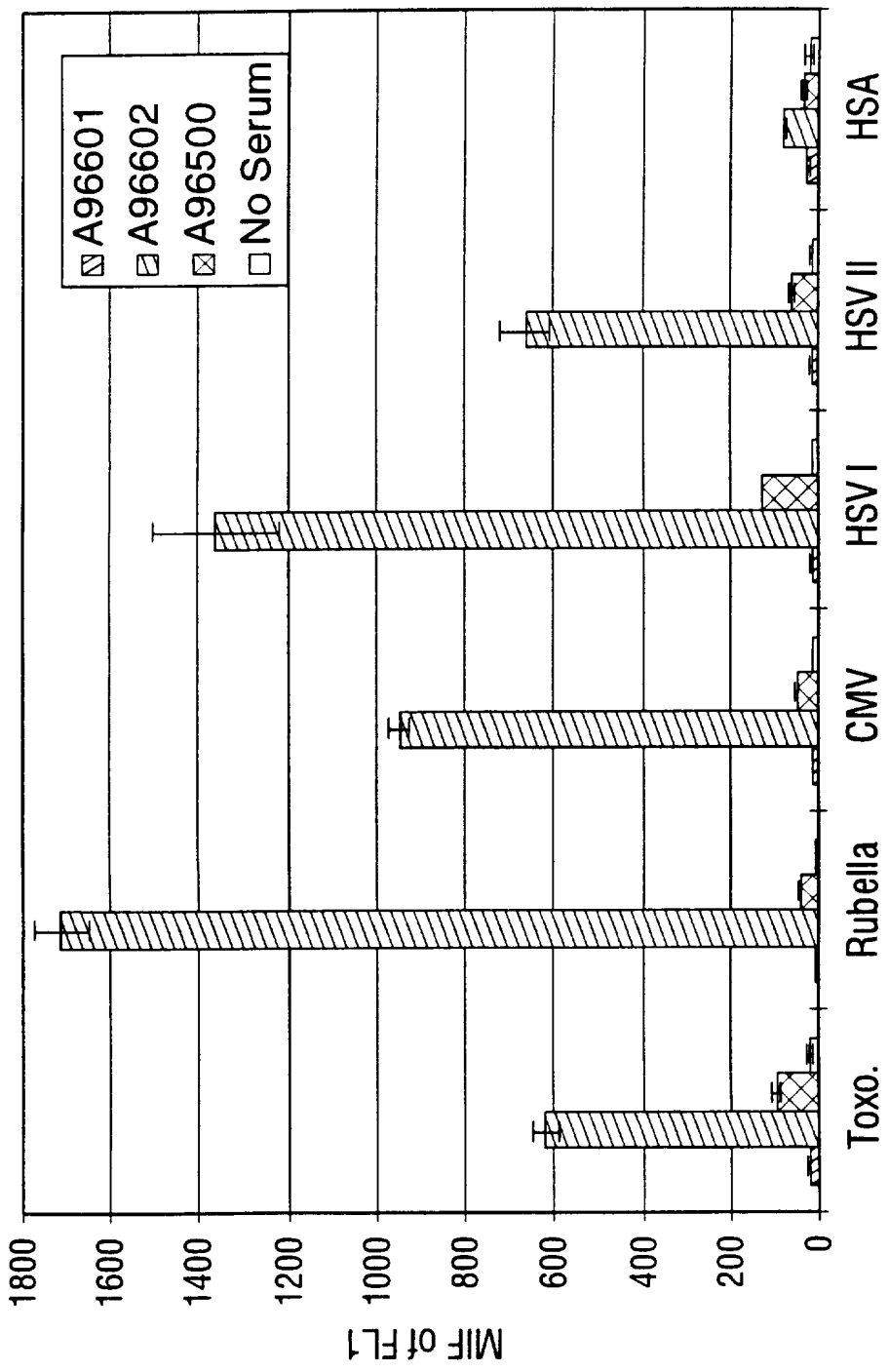

A96601
A96602
A96500
No Serum
MIF of FL1
1800
1600
1400
1200
1000
800
600
400
200
0
Toxo.
Rubella
CMV
HSV I
HSV II
HSA
ToRCH Antigen

FIG. 26A

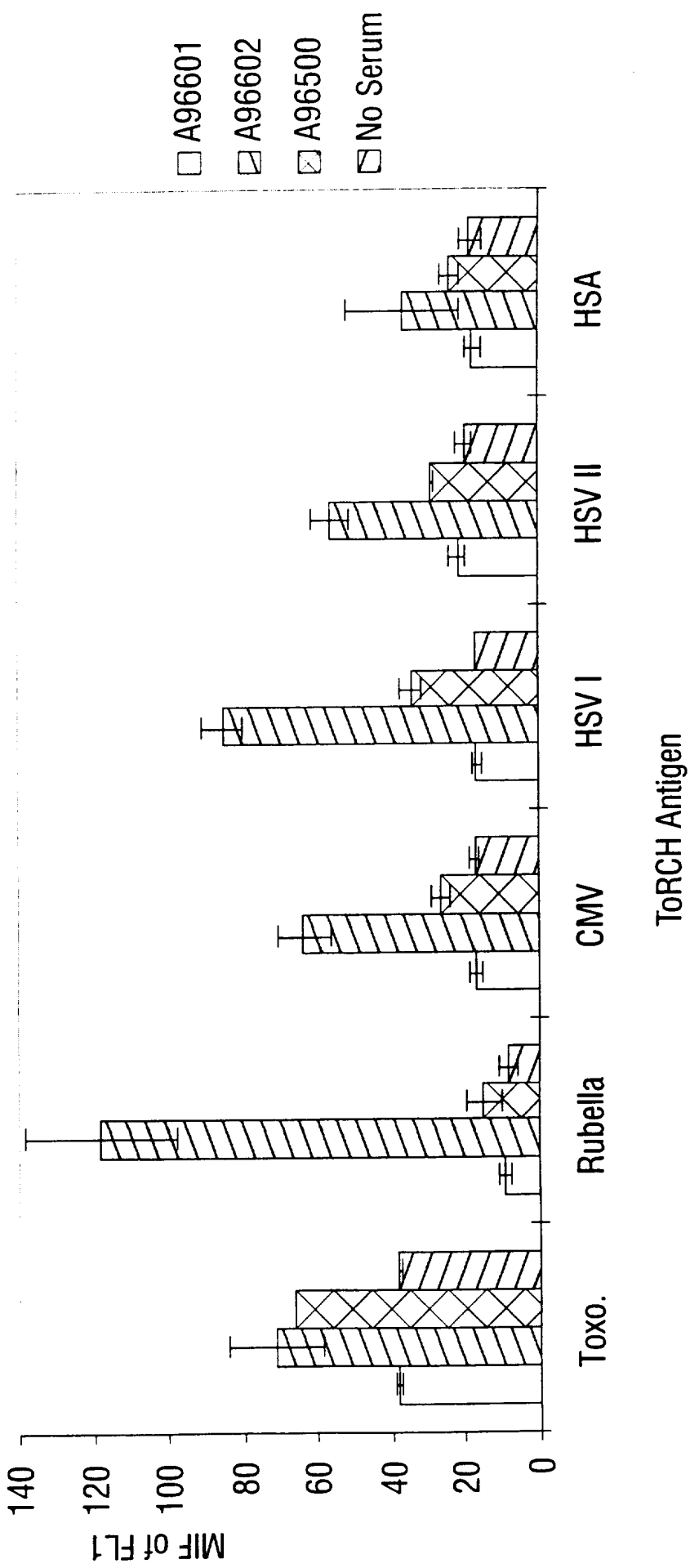

A96601
A96602
A96500
No Serum
MIF of FL1
0
20
40
60
80
100
120
140
Toxo.
Rubella
CMV
HSV I
HSV II
HSA
ToRCH Antigen

FIG. 26B

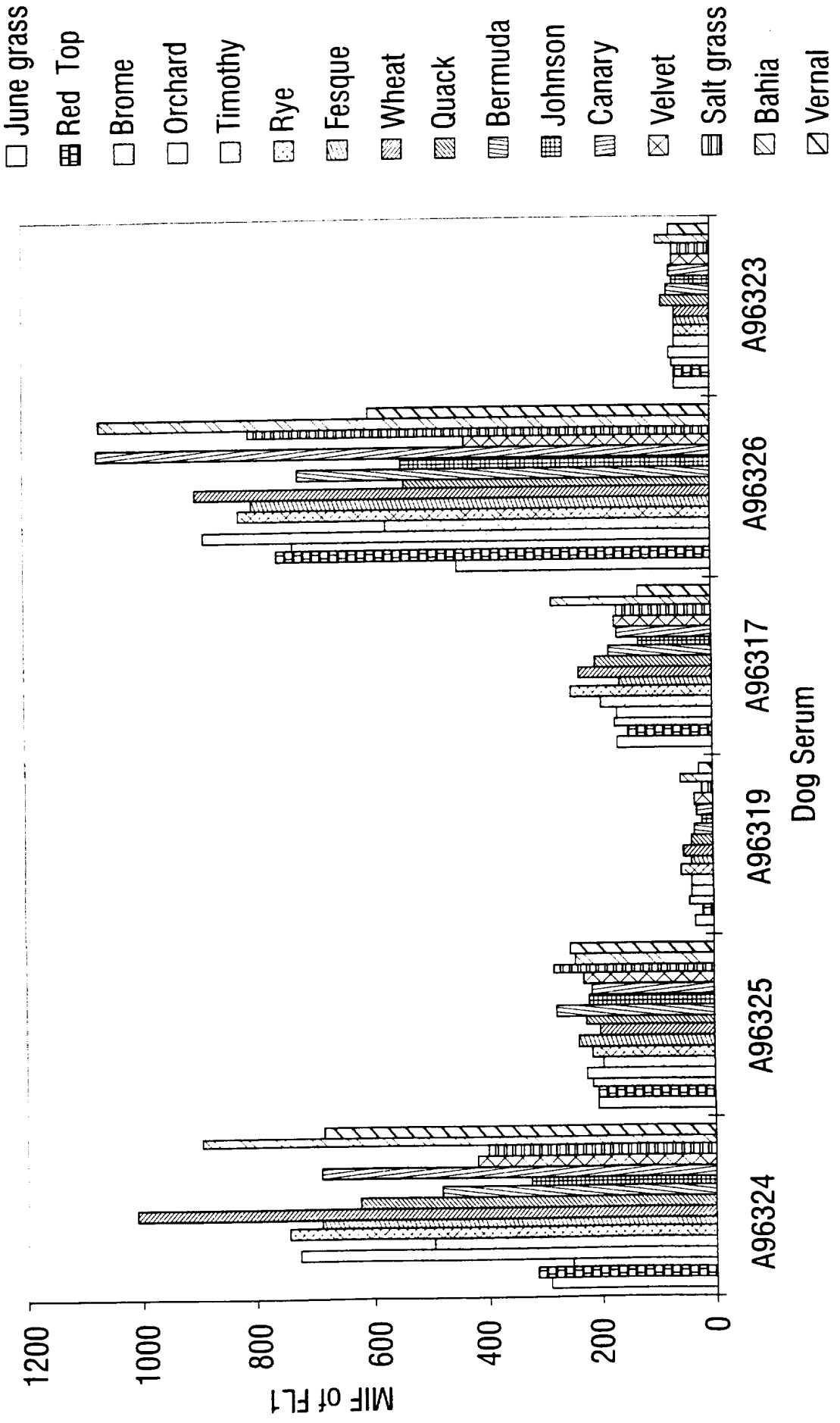

June grass
Red Top
Brome
Orchard
Timothy
Rye
Fesque
Wheat
Quack
Bermuda
Johnson
Canary
Velvet
Salt grass
Bahia
Vernal
MIF of FL1
0
200
400
600
800
1000
1200
A96324
A96325
A96319
A96317
A96326
A96323
Dog Serum

FIG. 27

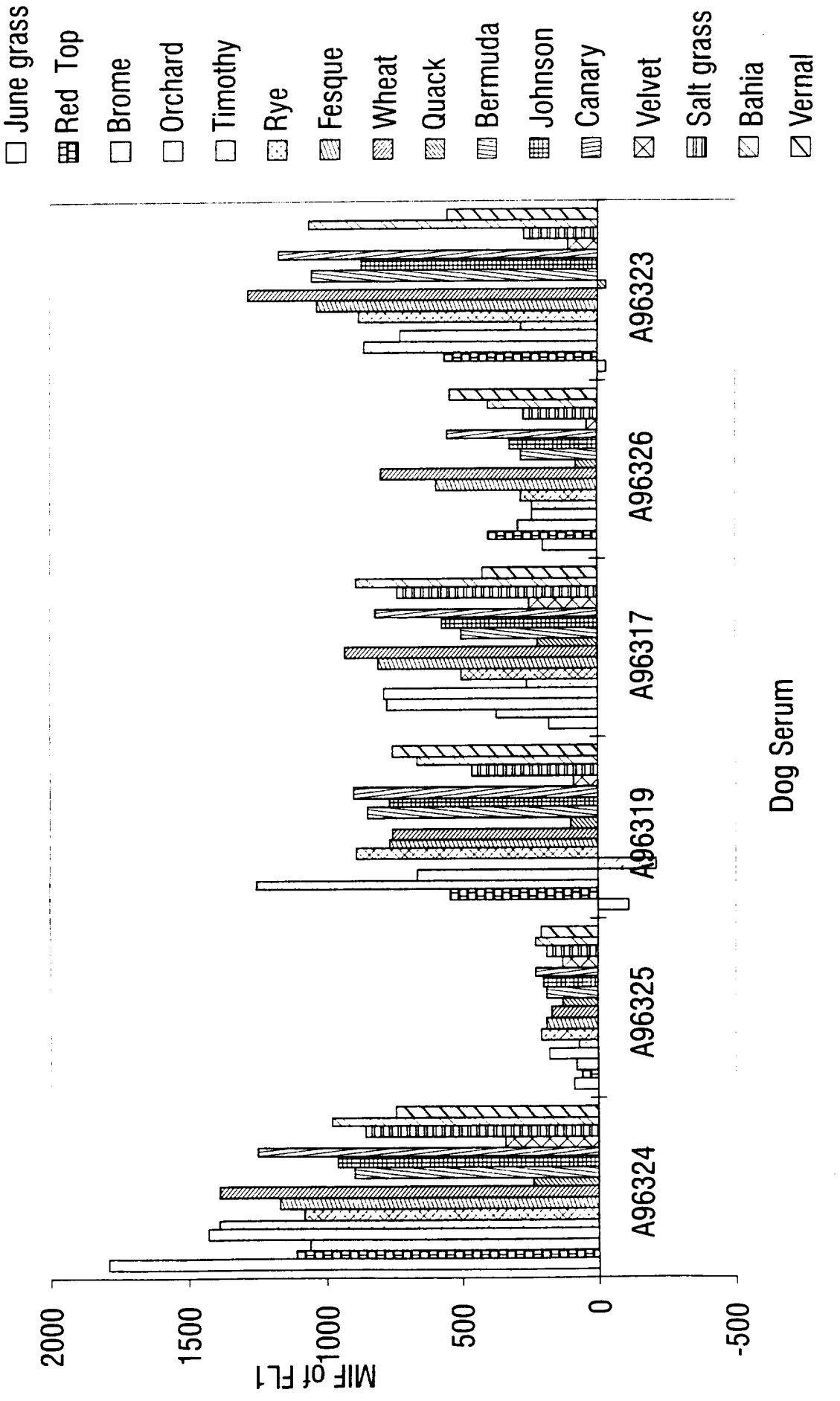

June grass
Red Top
Brome
Orchard
Timothy
Rye
Fesque
Wheat
Quack
Bermuda
Johnson
Canary
Velvet
Salt grass
Bahia
Vernal
2000
1500
1000
500
0
-500
MIF of FL1
A96324
A96325
A96319
A96317
A96326
A96323
Dog Serum

FIG. 28

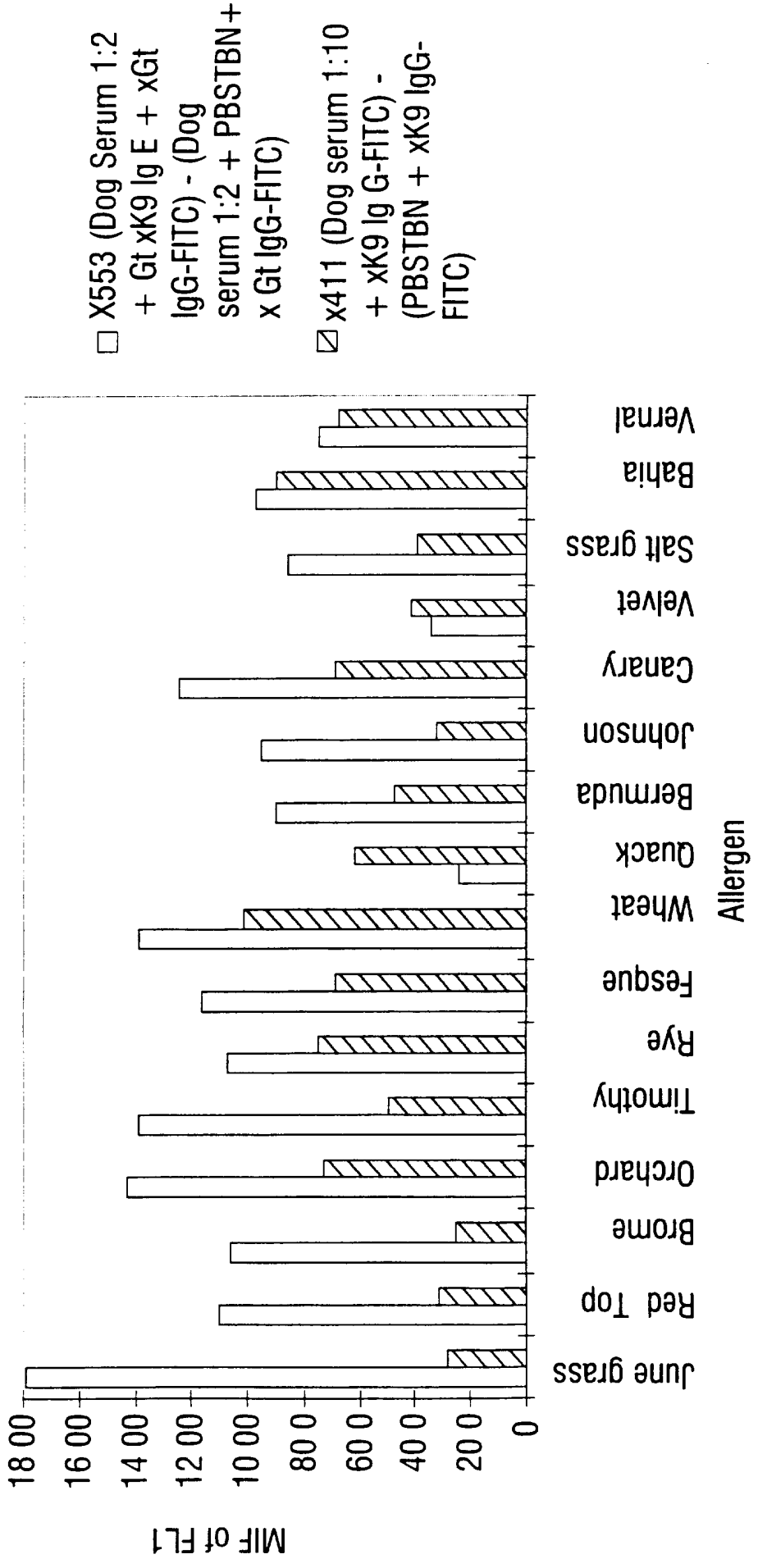

□ X553 (Dog Serum 1:2 + Gt xK9 Ig E + xGt IgG-FITC) - (Dog serum 1:2 + PBSTBN+ x Gt IgG-FITC)
☒ x411 (Dog serum 1:10 + xK9 Ig G-FITC) - (PBSTBN + xK9 IgG-FITC)
MIF of FL1
18 00
16 00
14 00
12 00
10 00
80 0
60 0
40 0
20 0
0
June grass
Red Top
Brome
Orchard
Timothy
Rye
Fesque
Wheat
Quack
Bermuda
Johnson
Canary
Velvet
Salt grass
Bahia
Vernal
Allergen

FIG. 29

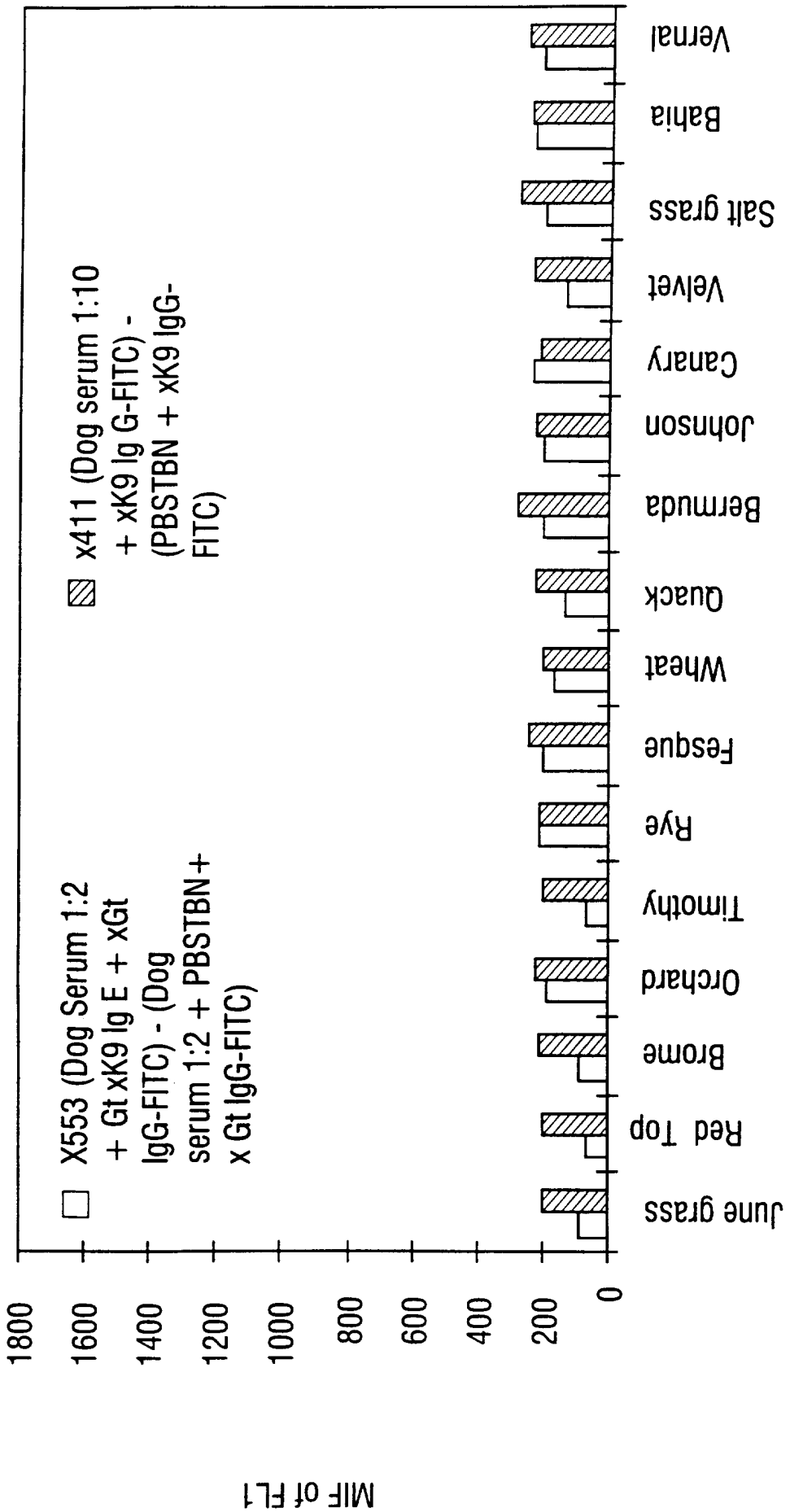

MIF of FL1
1800
1600
1400
1200
1000
800
600
400
200
0
X553 (Dog Serum 1:2 + Gt xK9 Ig E + xGt IgG-FITC) - (Dog serum 1:2 + PBSTBN+ x Gt IgG-FITC)
x411 (Dog serum 1:10 + xK9 Ig G-FITC) - (PBSTBN + xK9 IgG-FITC)
June grass
Red Top
Brome
Orchard
Timothy
Rye
Fesque
Wheat
Quack
Bermuda
Johnson
Canary
Velvet
Salt grass
Bahia
Vernal
Allergen

FIG. 30

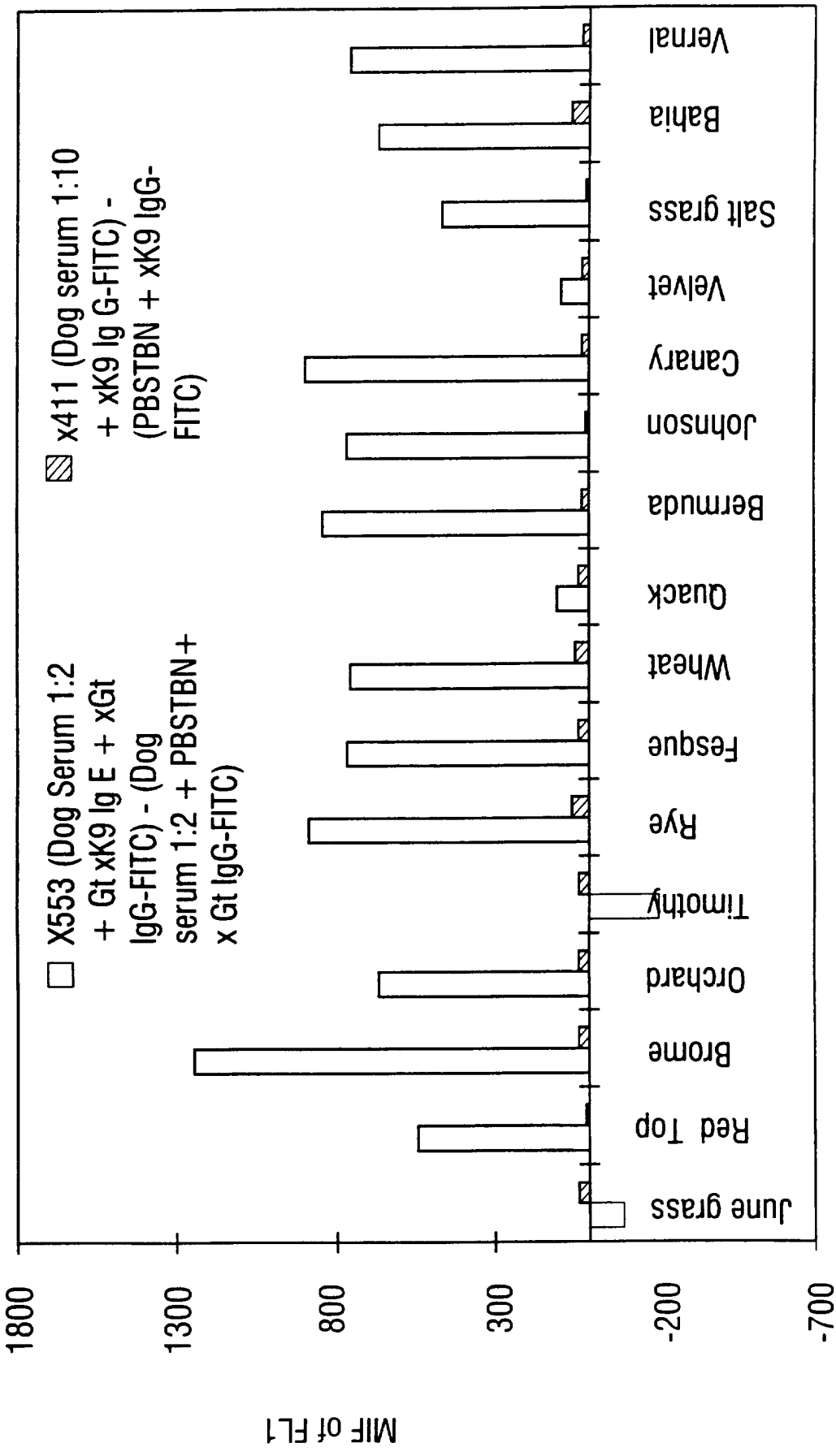

MIF of FL1
1800
1300
800
300
-200
-700
June grass
Red Top
Brome
Orchard
Timothy
Rye
Fesque
Wheat
Quack
Bermuda
Johnson
Canary
Velvet
Salt grass
Bahia
Vernal
Allergen
X553 (Dog Serum 1:2 + Gt xK9 Ig E + xGt IgG-FITC) - (Dog serum 1:2 + PBSTBN+ x Gt IgG-FITC)
x411 (Dog serum 1:10 + xK9 Ig G-FITC) - (PBSTBN + xK9 IgG-FITC)

FIG. 31

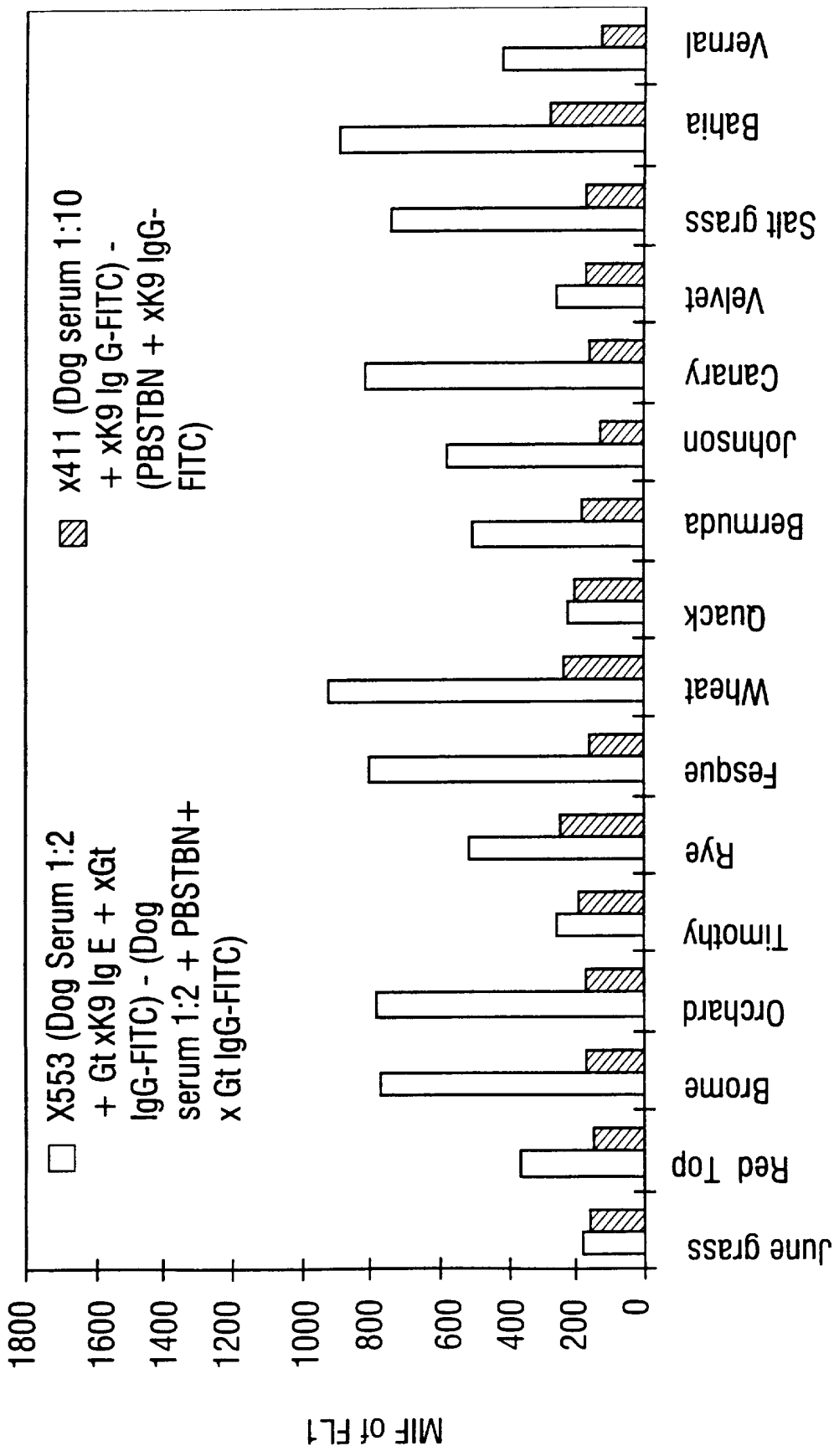

MIF of FL1
1800
1600
1400
1200
1000
800
600
400
200
0
X553 (Dog Serum 1:2 + Gt xK9 Ig E + xGt IgG-FITC) - (Dog serum 1:2 + PBSTBN + x Gt IgG-FITC)
x411 (Dog serum 1:10 + xK9 Ig G-FITC) - (PBSTBN + xK9 IgG-FITC)
June grass
Red Top
Brome
Orchard
Timothy
Rye
Fesque
Wheat
Quack
Bermuda
Johnson
Canary
Velvet
Salt grass
Bahia
Vernal
Allergen

FIG. 32

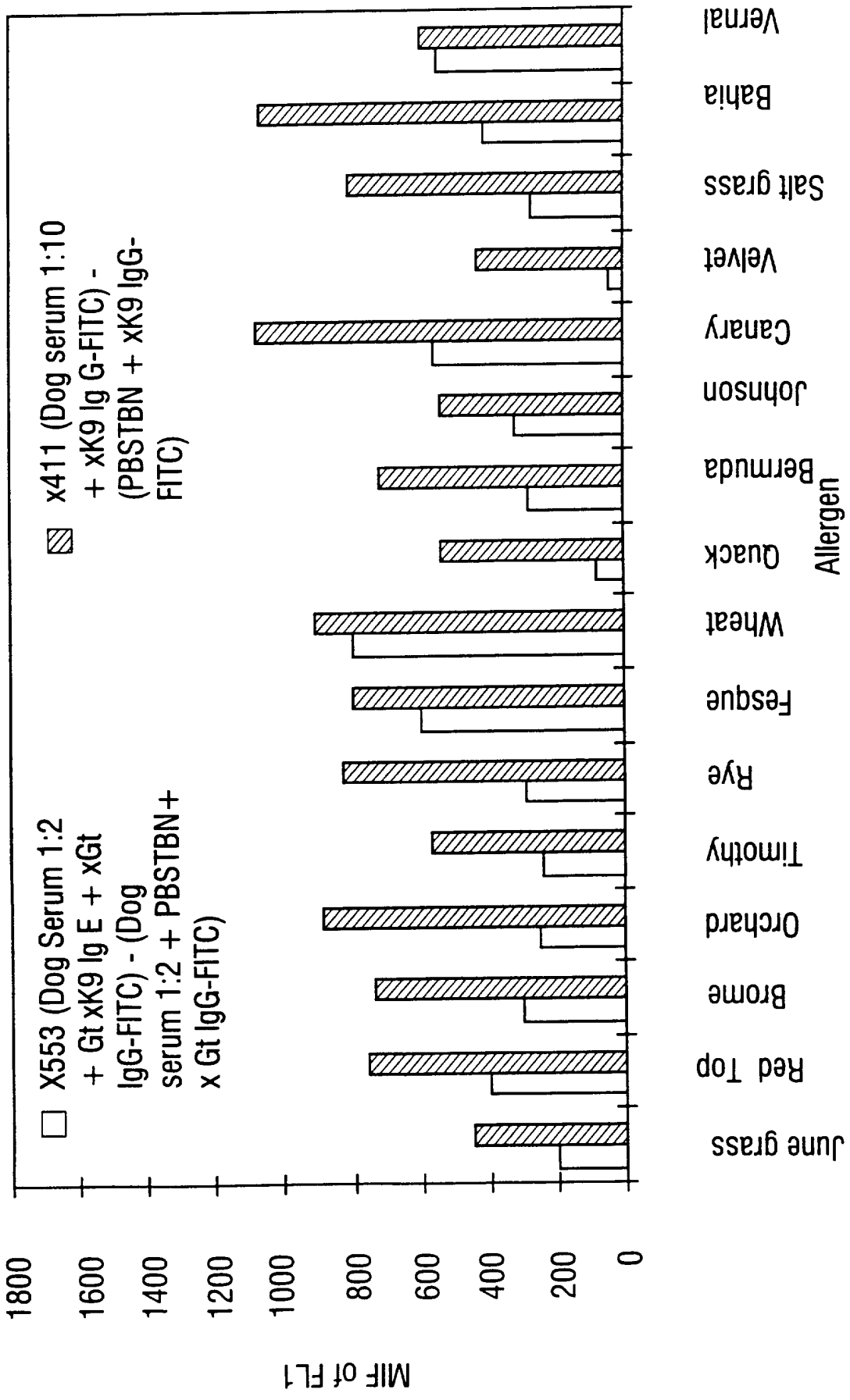

MIF of FL1
1800
1600
1400
1200
1000
800
600
400
200
0
X553 (Dog Serum 1:2 + Gt xK9 Ig E + xGt IgG-FITC) - (Dog serum 1:2 + PBSTBN+ x Gt IgG-FITC)
x411 (Dog serum 1:10 + xK9 Ig G-FITC) - (PBSTBN + xK9 IgG-FITC)
June grass
Red Top
Brome
Orchard
Timothy
Rye
Fesque
Wheat
Quack
Bermuda
Johnson
Canary
Velvet
Salt grass
Bahia
Vernal
Allergen

FIG. 33

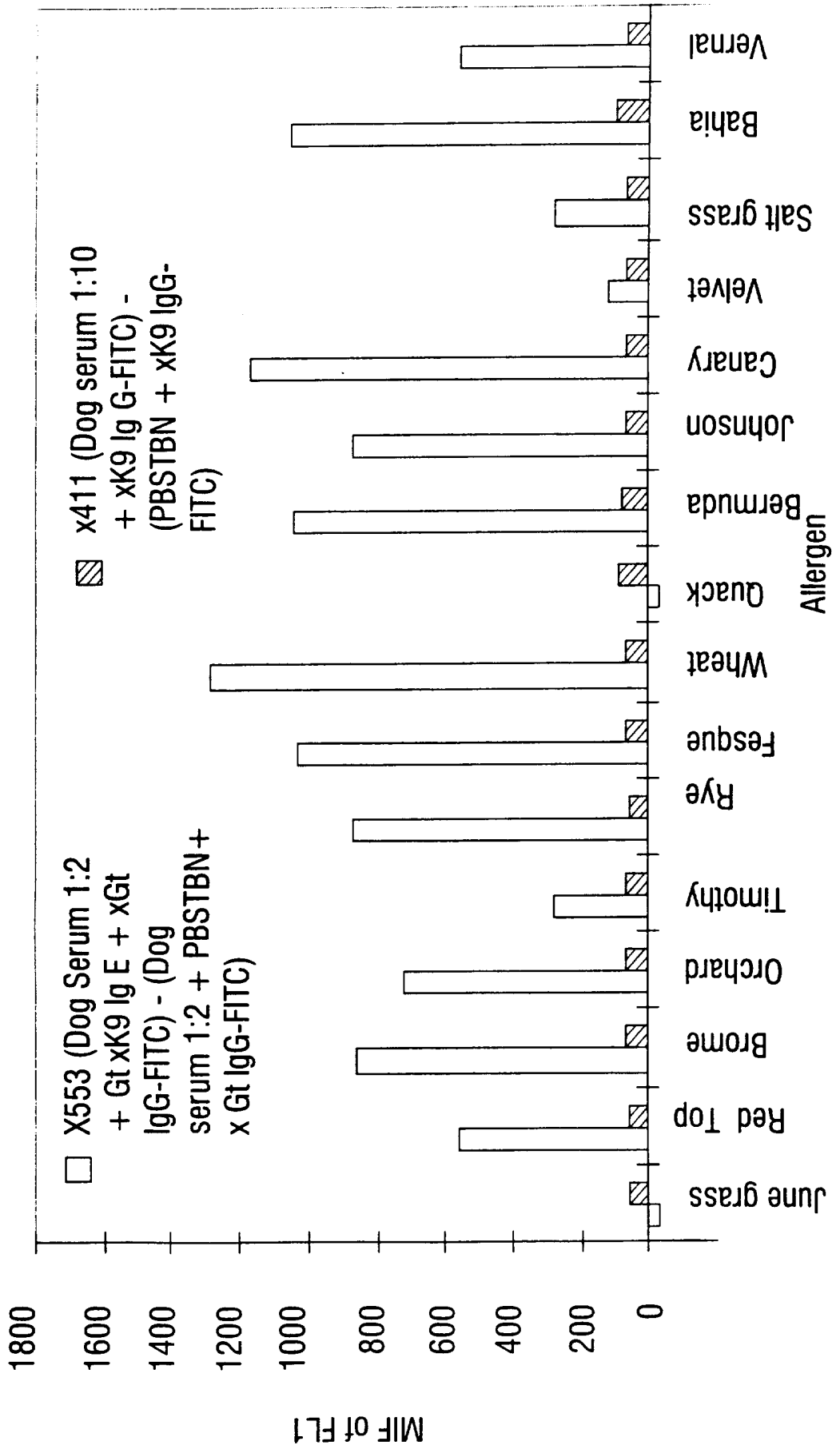

MIF of FL1
1800
1600
1400
1200
1000
800
600
400
200
0
June grass
Red Top
Brome
Orchard
Timothy
Rye
Fesque
Wheat
Quack
Bermuda
Johnson
Canary
Velvet
Salt grass
Bahia
Vernal
Allergen
X553 (Dog Serum 1:2 + Gt xK9 Ig E + xGt IgG-FITC) - (Dog serum 1:2 + PBSTBN+ x Gt IgG-FITC)
x411 (Dog serum 1:10 + xK9 Ig G-FITC) - (PBSTBN + xK9 IgG-FITC)

FIG. 34

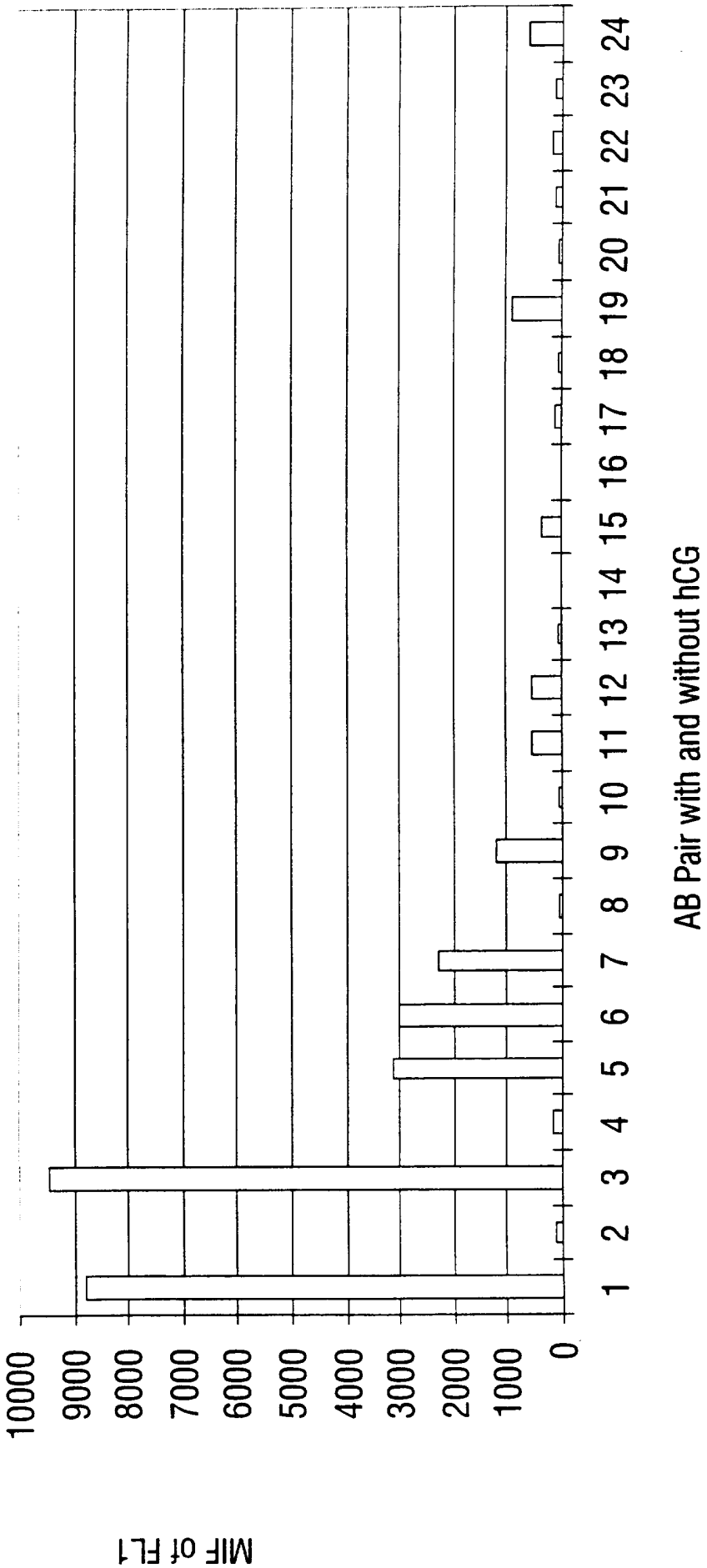

MIF of FL1
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
AB Pair with and without hCG

FIG. 35

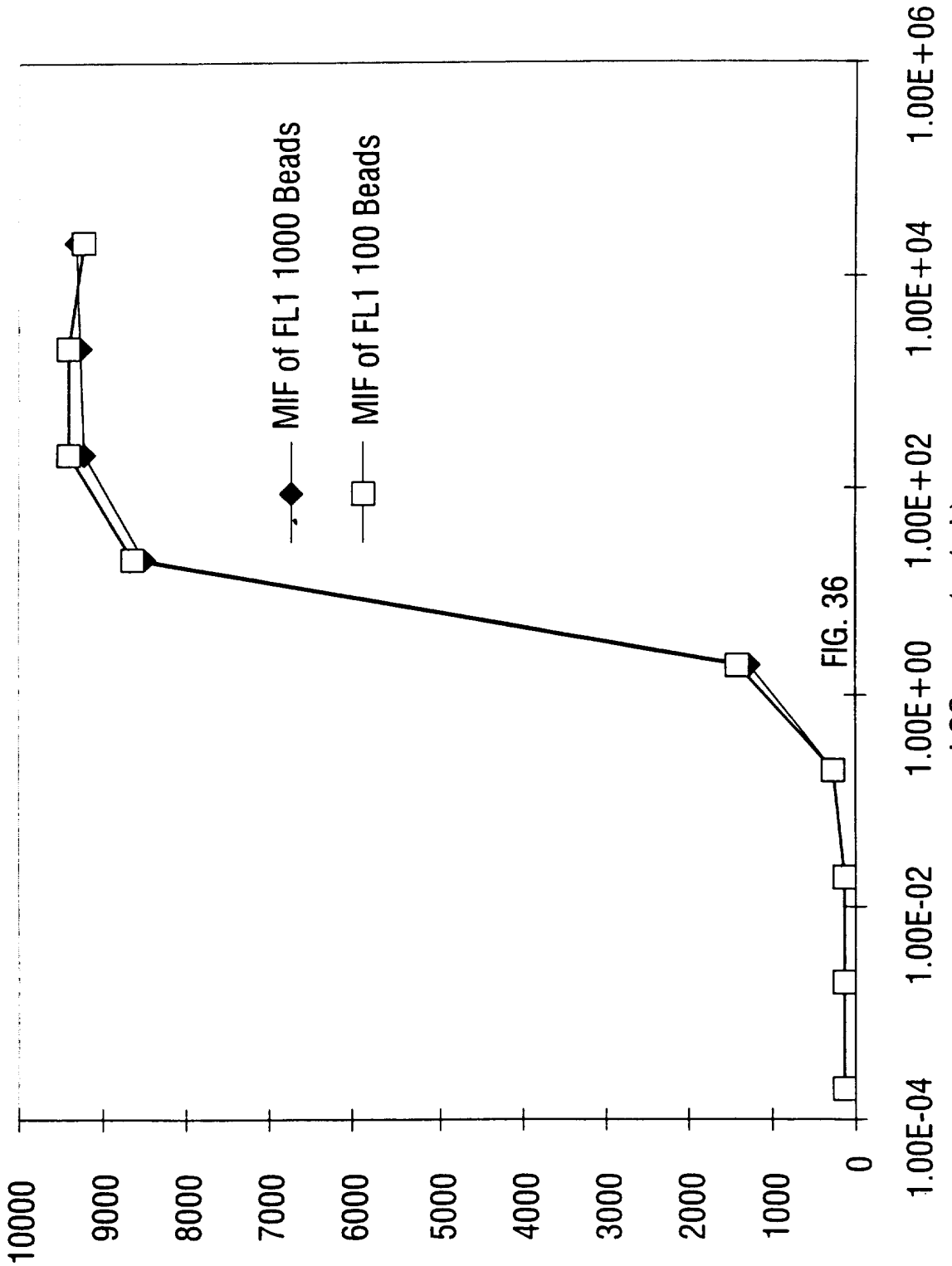

MIF of FL1 1000 Beads
MIF of FL1 100 Beads
MIF of FL1
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
1.00E-04
1.00E-02
1.00E+00
1.00E+02
1.00E+04
1.00E+06
hCG conc. (ng/mL)
FIG. 36

FIG. 36

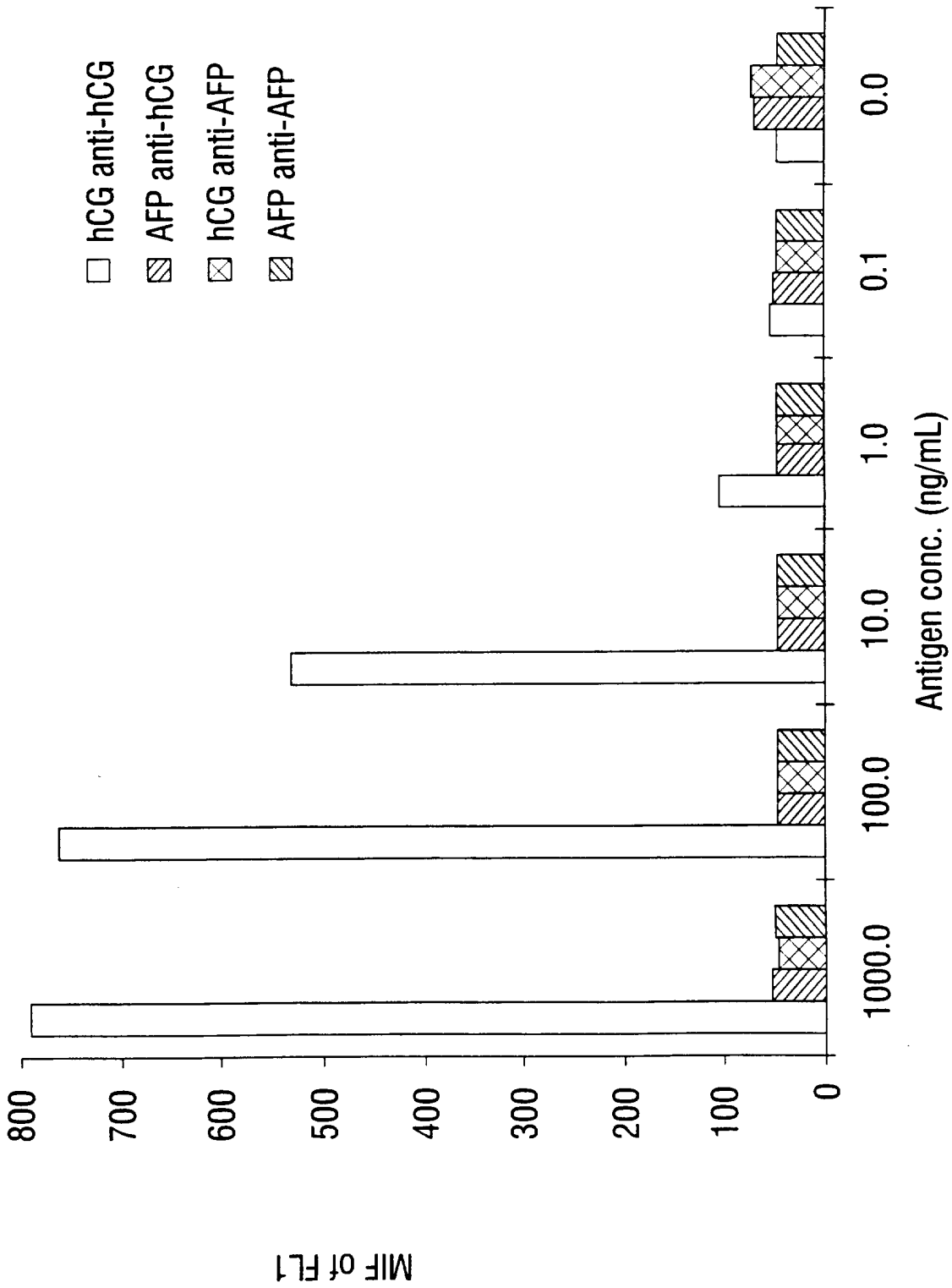

hCG anti-hCG
AFP anti-hCG
hCG anti-AFP
AFP anti-AFP
MIF of FL1
800
700
600
500
400
300
200
100
0
1000.0
100.0
10.0
1.0
0.1
0.0
Antigen conc. (ng/mL)

FIG. 37A

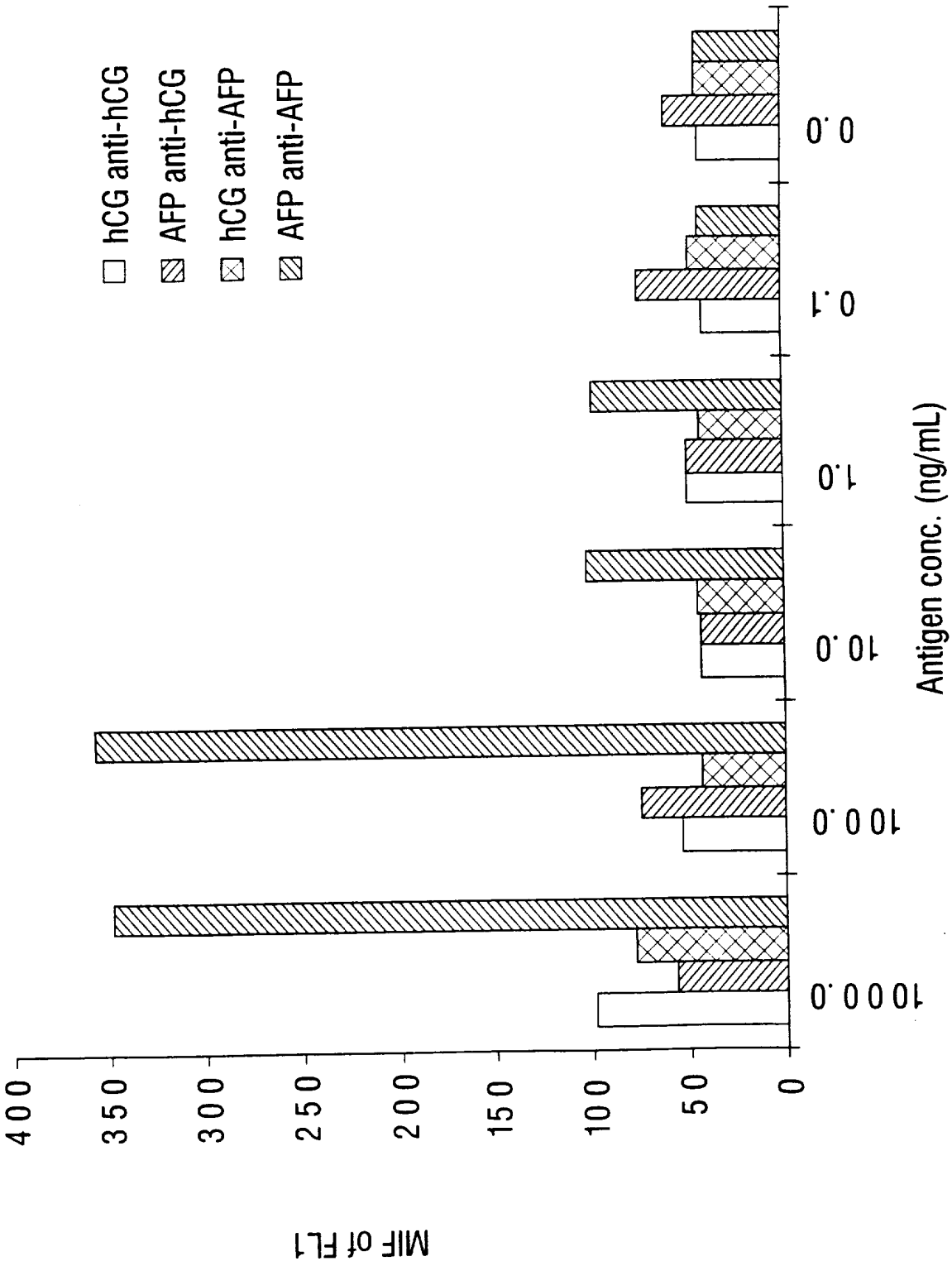

hCG anti-hCG
AFP anti-hCG
hCG anti-AFP
AFP anti-AFP
MIF of FL1
400
350
300
250
200
150
100
50
0
10000.0
1000.0
100.0
10.0
1.0
0.1
0.0
Antigen conc. (ng/mL)

FIG. 37B

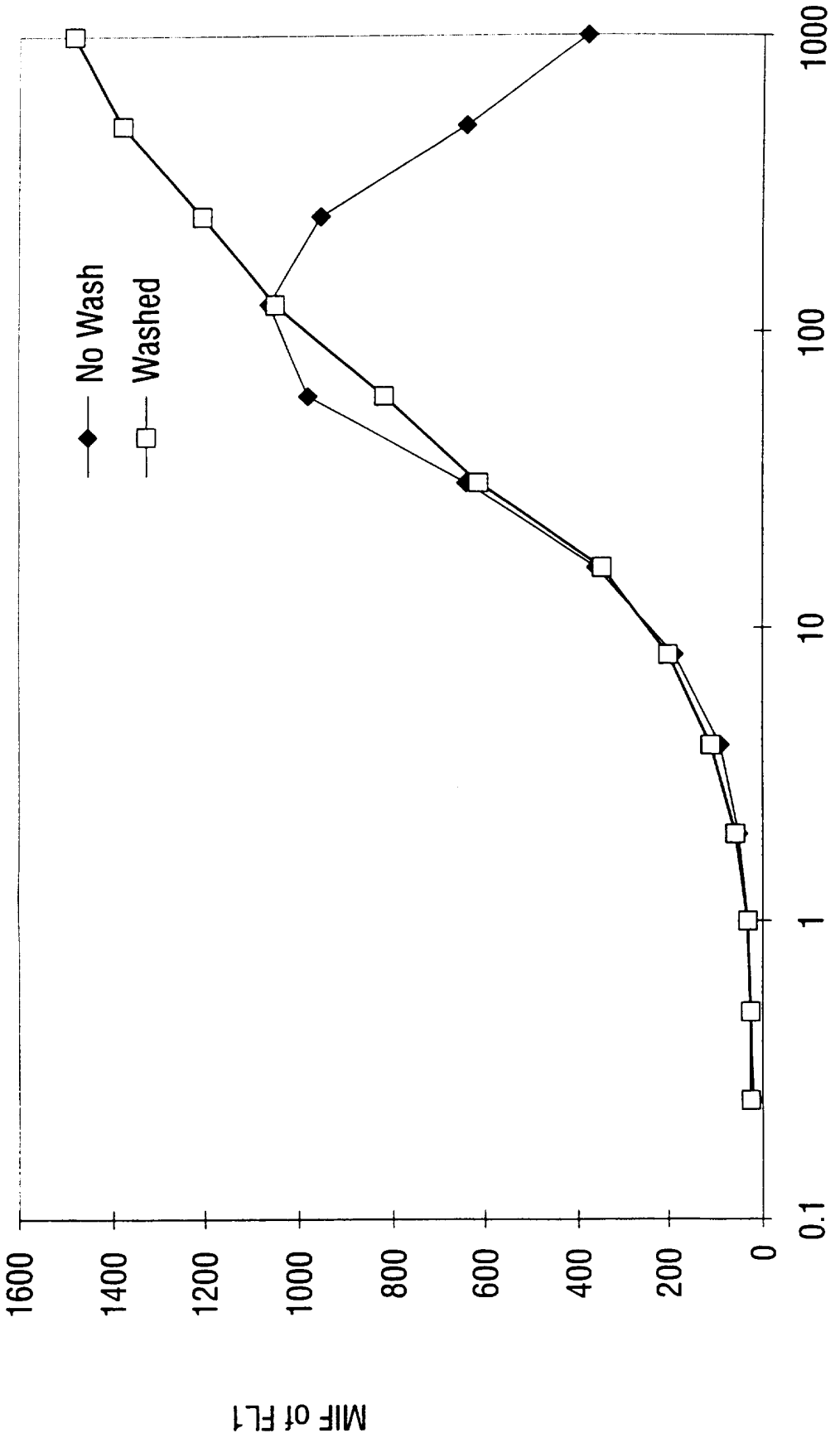

No Wash
Washed
MIF of FL1
1600
1400
1200
1000
800
600
400
200
0
0.1
1
10
100
1000
AFP Conc. (ng/mL)

FIG. 38A

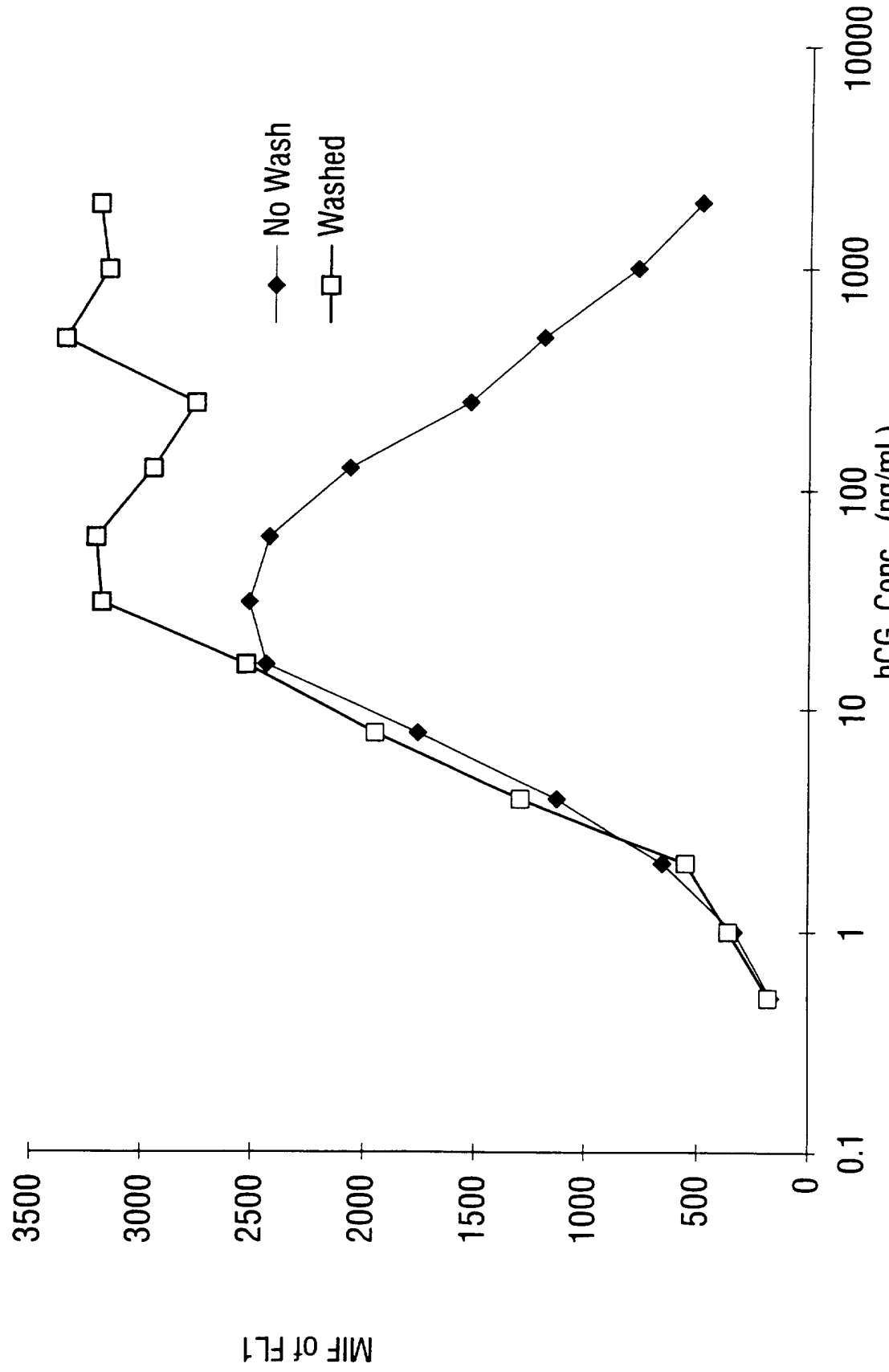

MIF of FL1
3500
3000
2500
2000
1500
1000
500
0
0.1
1
10
100
1000
10000
hCG Conc. (ng/mL)
No Wash
Washed

FIG. 38B

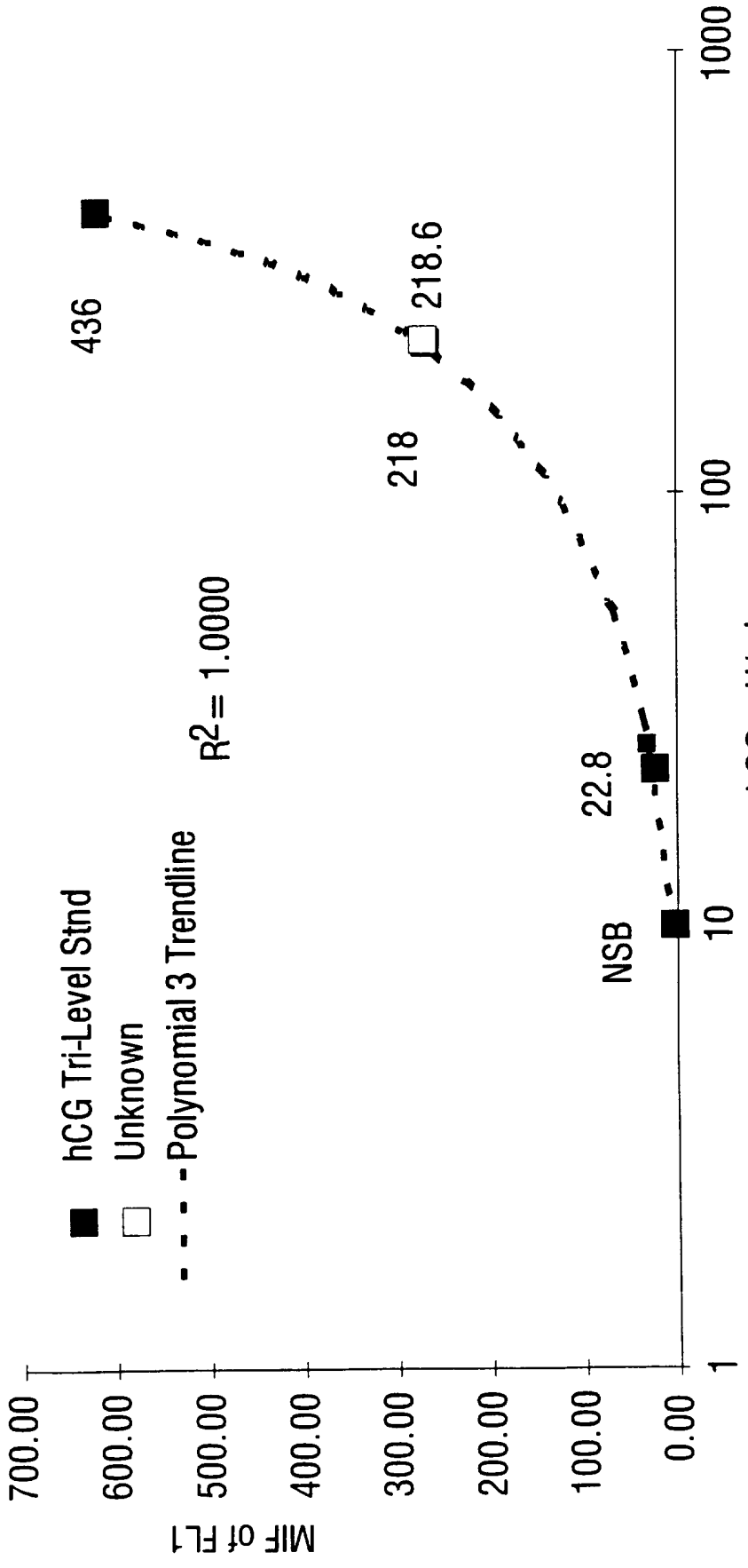

700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
MIF of FL1
1
10
100
1000
hCG mU/ml
NSB
22.8
218
218.6
436
R2= 1.0000
hCG Tri-Level Stnd
Unknown
Polynomial 3 Trendline

FIG. 39A

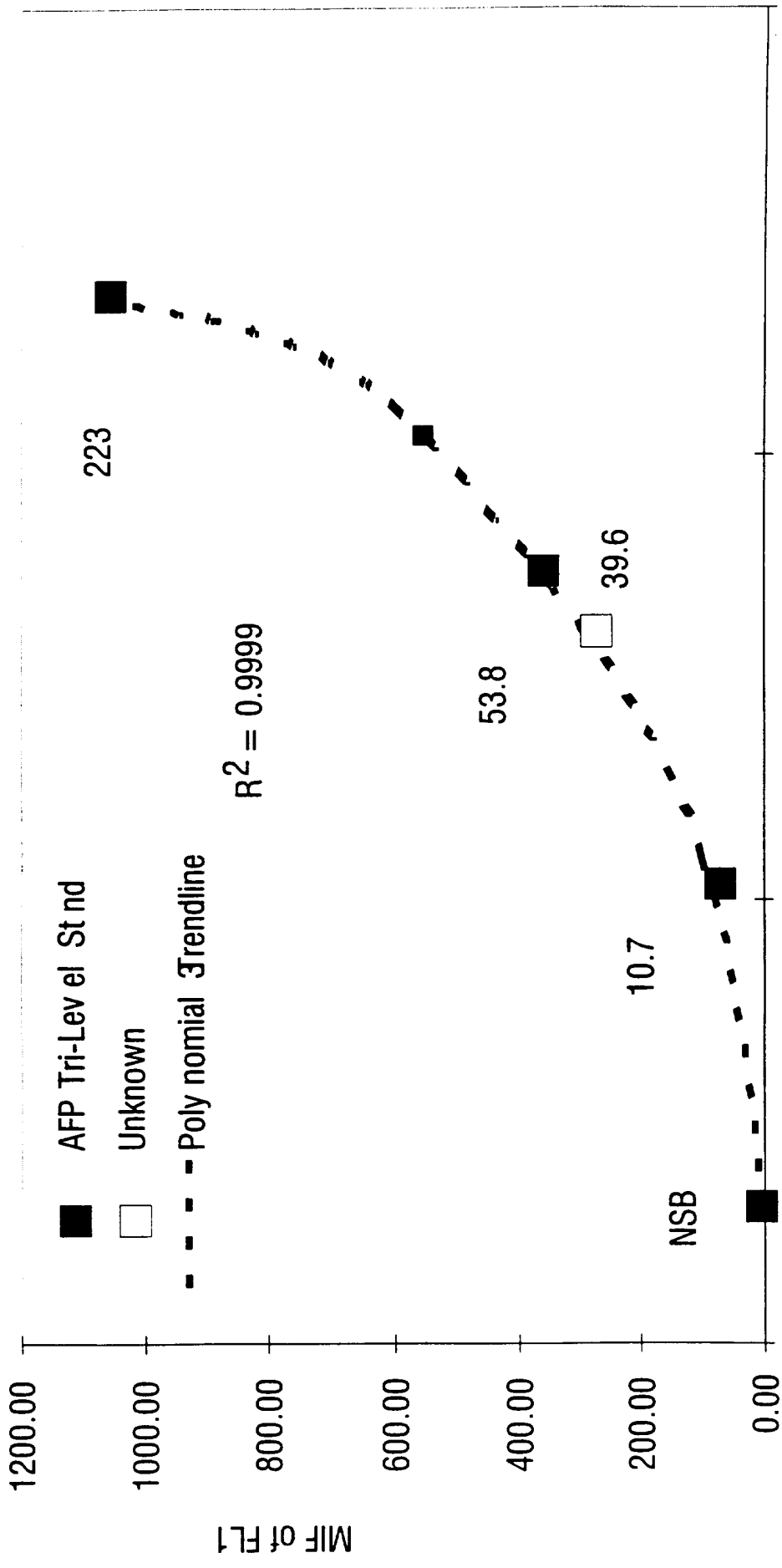

MIF of FL1
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
AFP Tri-Level Stnd
Unknown
Polynomial Trendline
$R^2 = 0.9999$
223
53.8
39.6
10.7
NSB

FIG. 39B

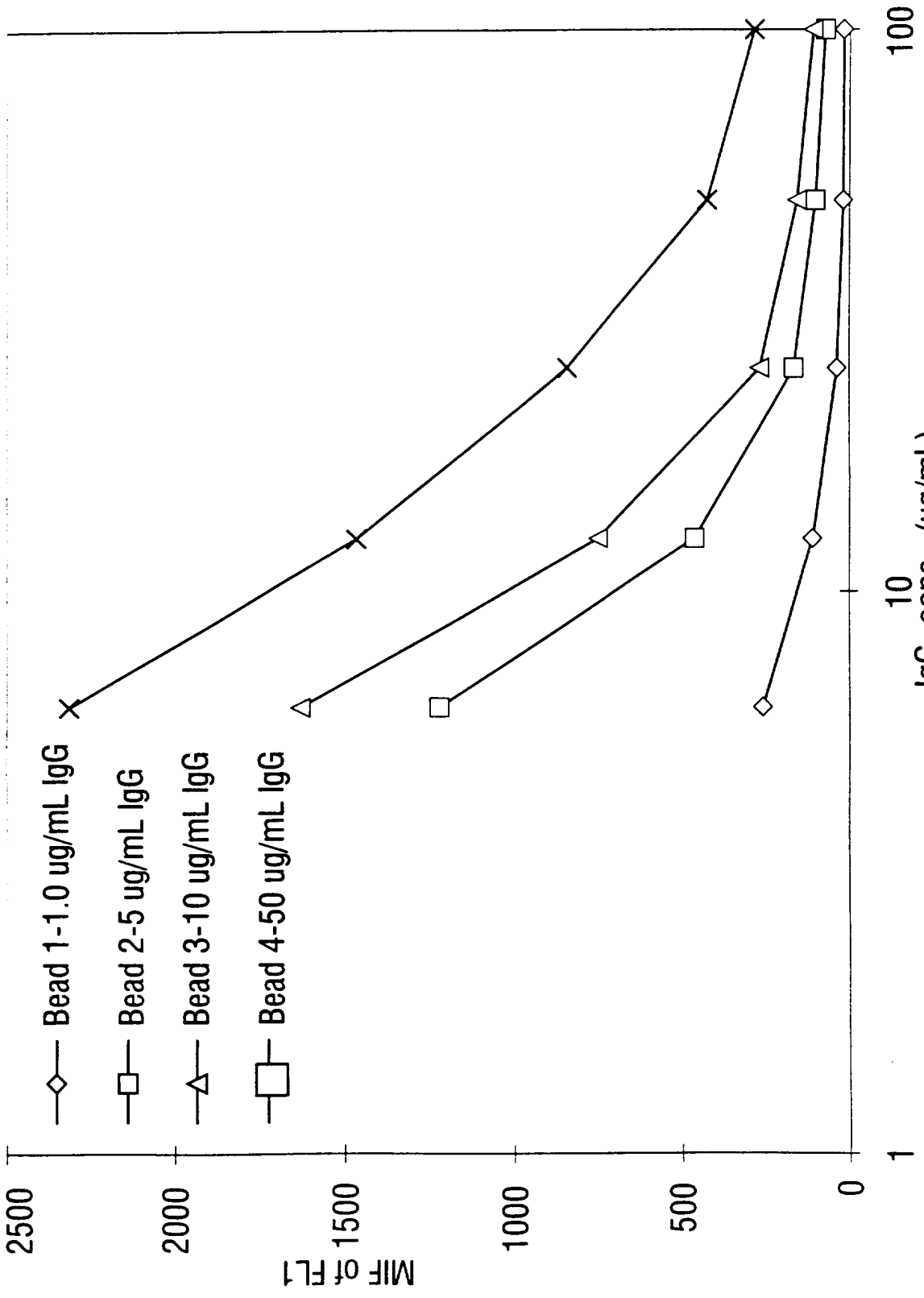

MIF of FL1
2500
2000
1500
1000
500
0
1
10
100
IgG- conc. (ug/mL)
Bead 1-1.0 ug/mL IgG
Bead 2-5 ug/mL IgG
Bead 3-10 ug/mL IgG
Bead 4-50 ug/mL IgG

FIG. 40

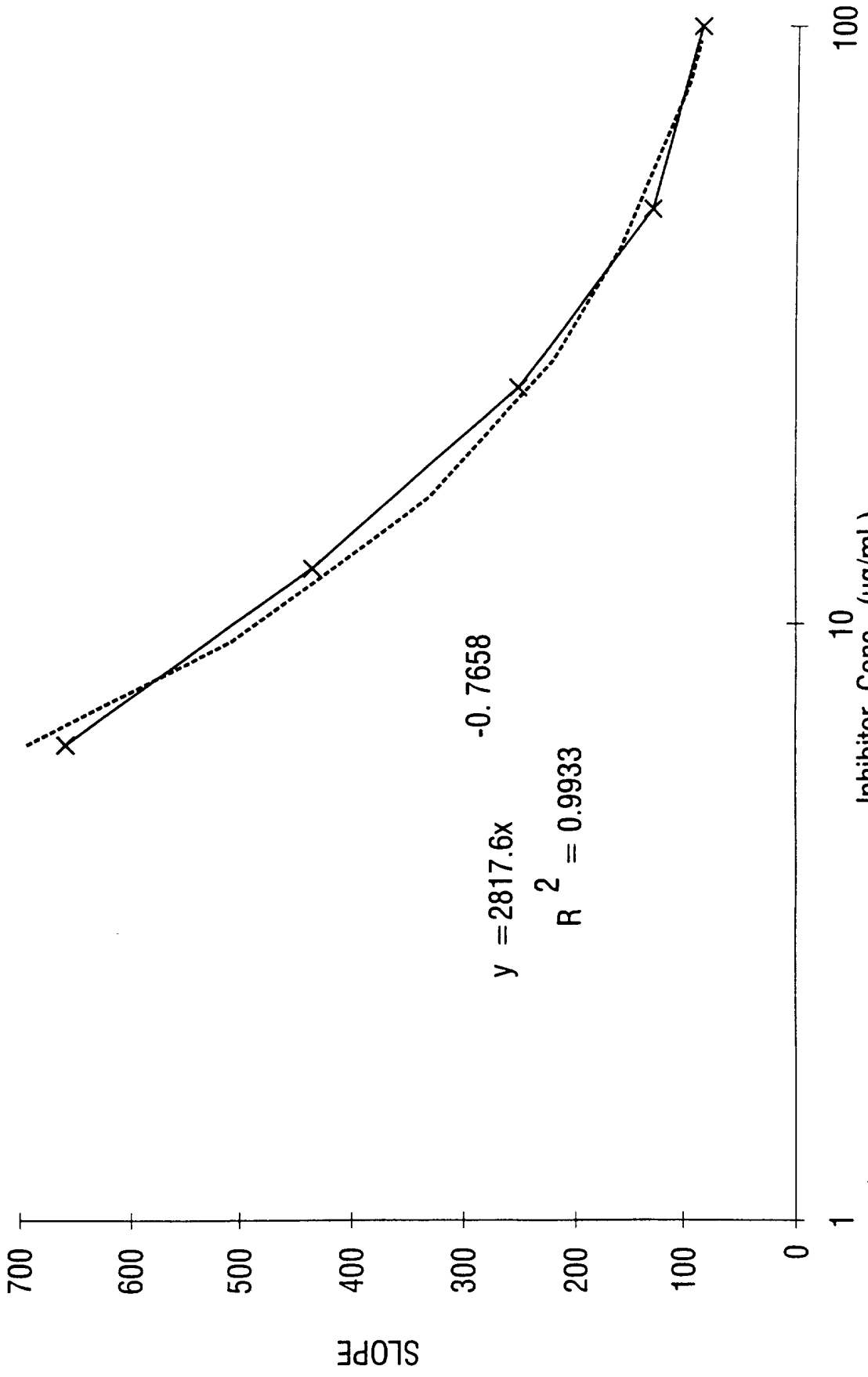

SLOPE
700
600
500
400
300
200
100
0
1
10
100
Inhibitor Conc. (ug/mL)
y = 2817.6x - 0.7658
R 2 = 0.9933

FIG. 41

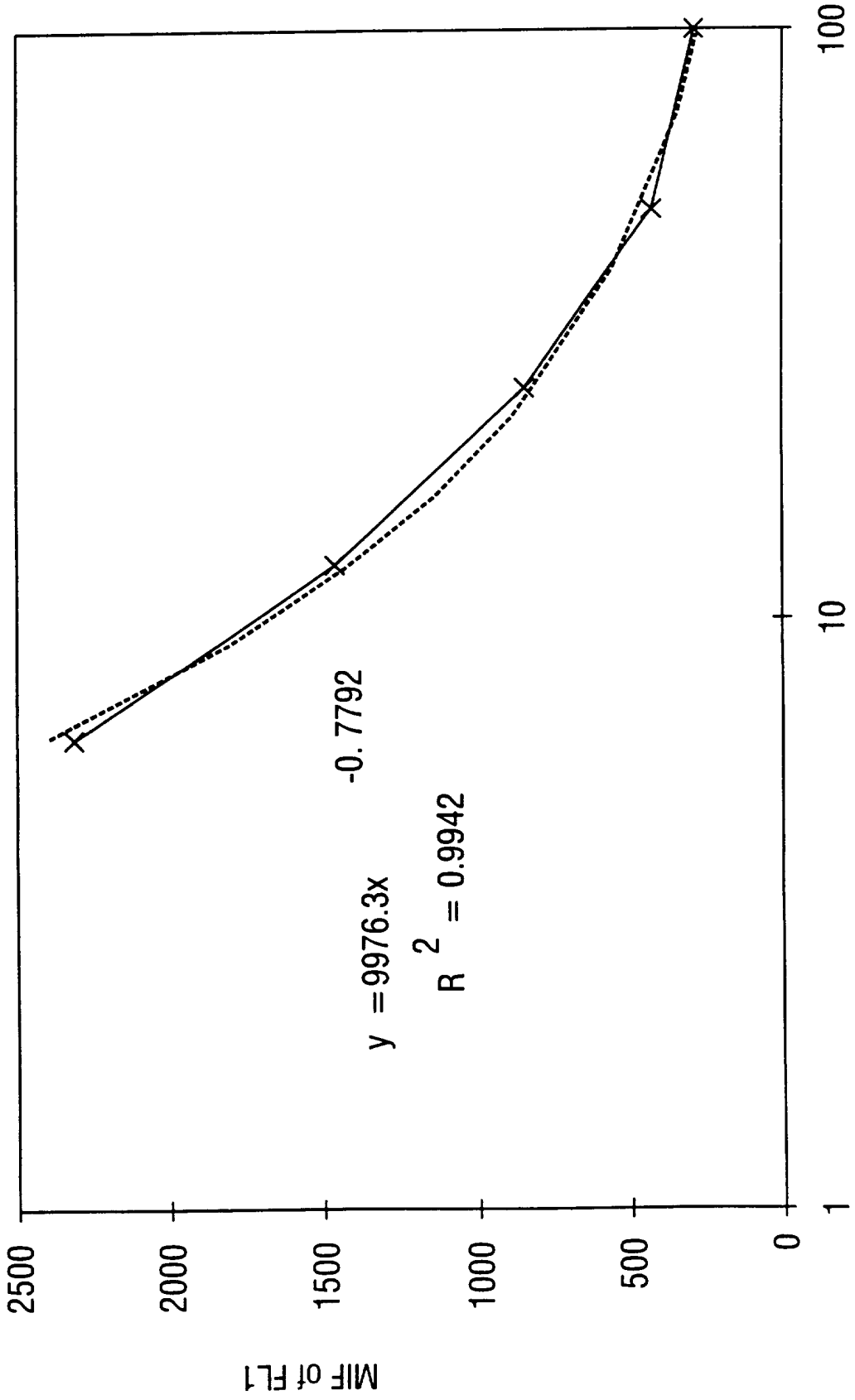

MIF of FL1
2500
2000
1500
1000
500
0
1
10
100
hIgG Conc. (ug/mL)
y =9976.3x -0. 7792
R 2 = 0.9942

FIG. 42

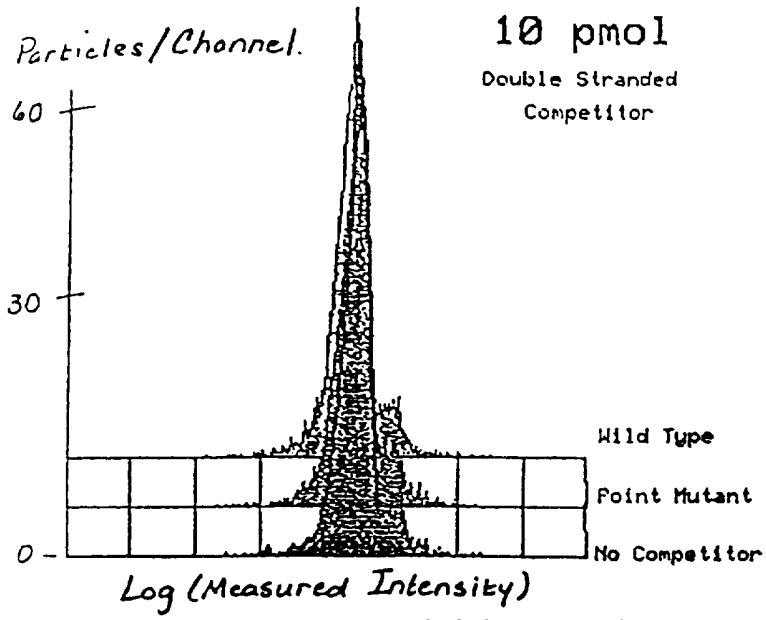


Fig. 43a

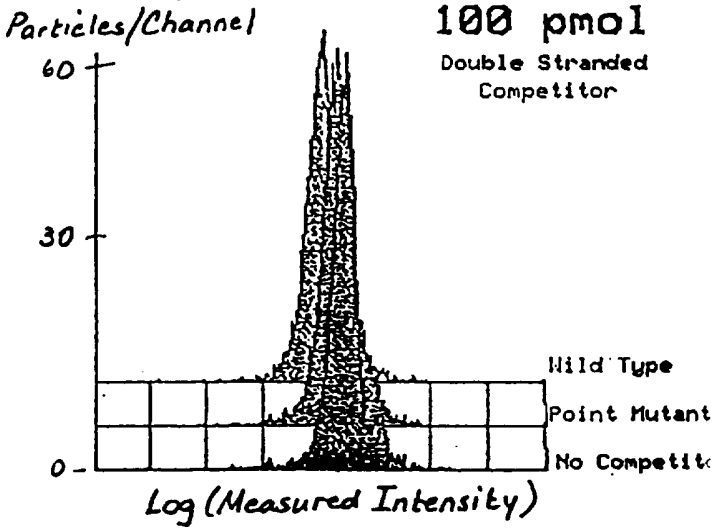


Fig. 43b

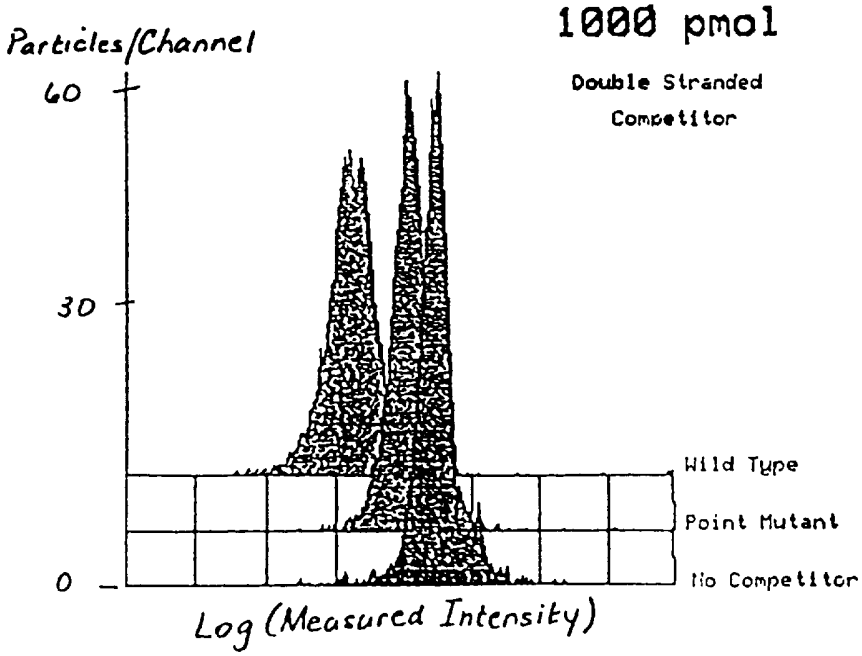


Fig. 43c

Fig. 44a

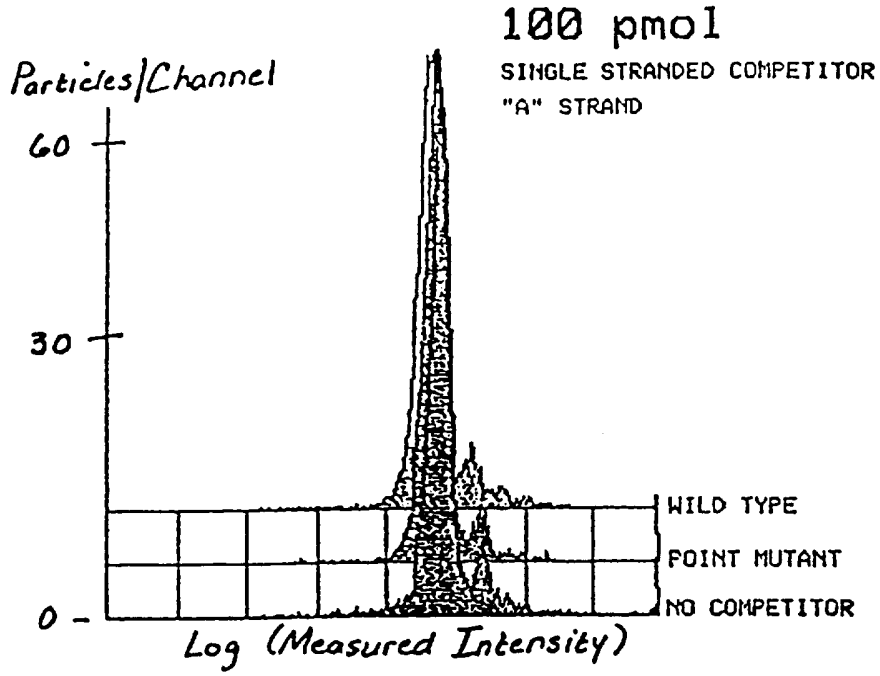


Fig. 44b

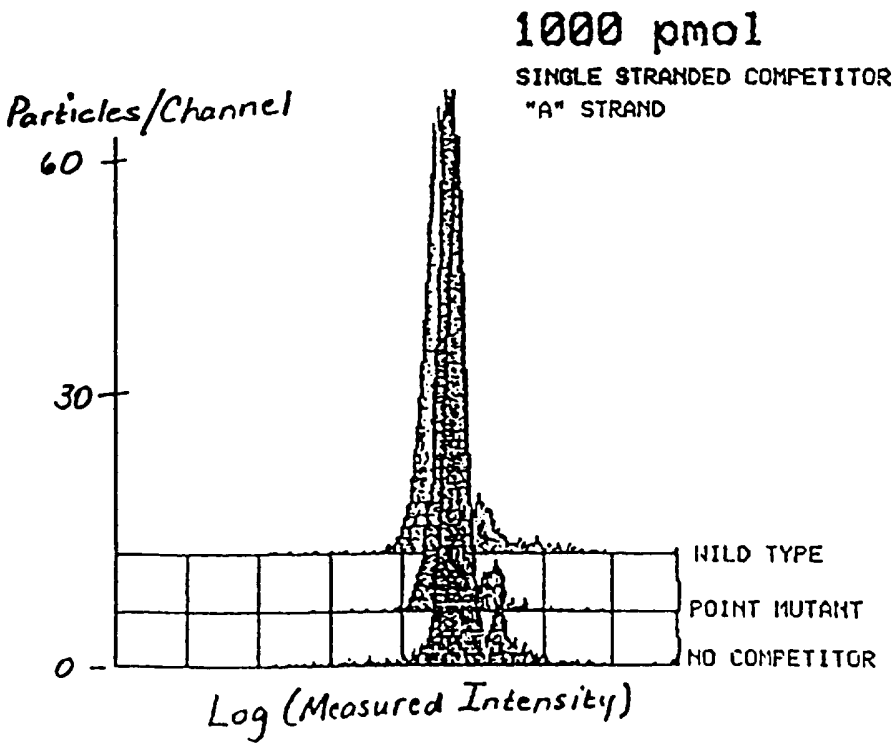


Fig. 45

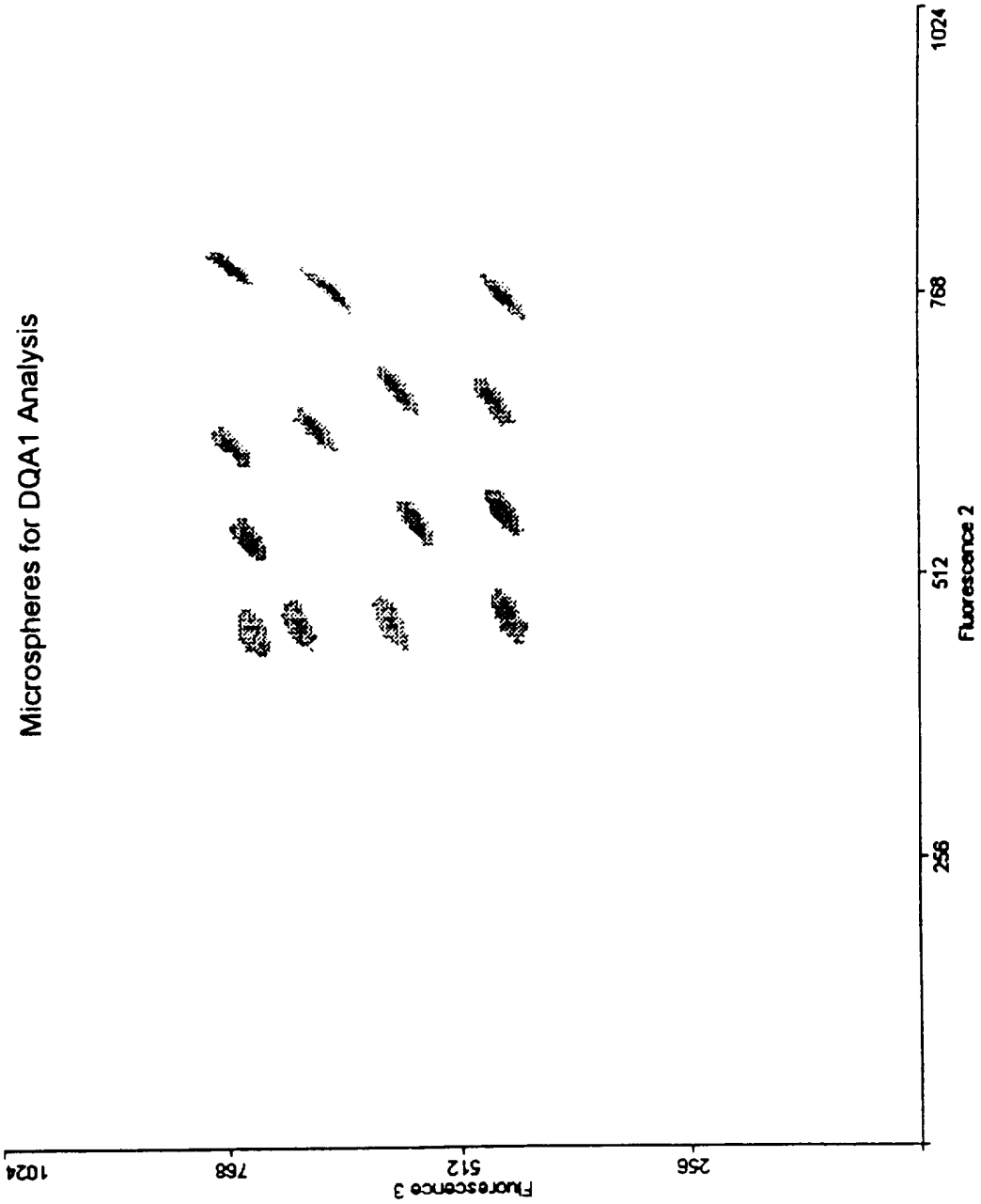

Microspheres for DQA1 Analysis
Fluorescence 3
256
512
768
1024
Fluorescence 2
256
512
768
1024

Fig. 46

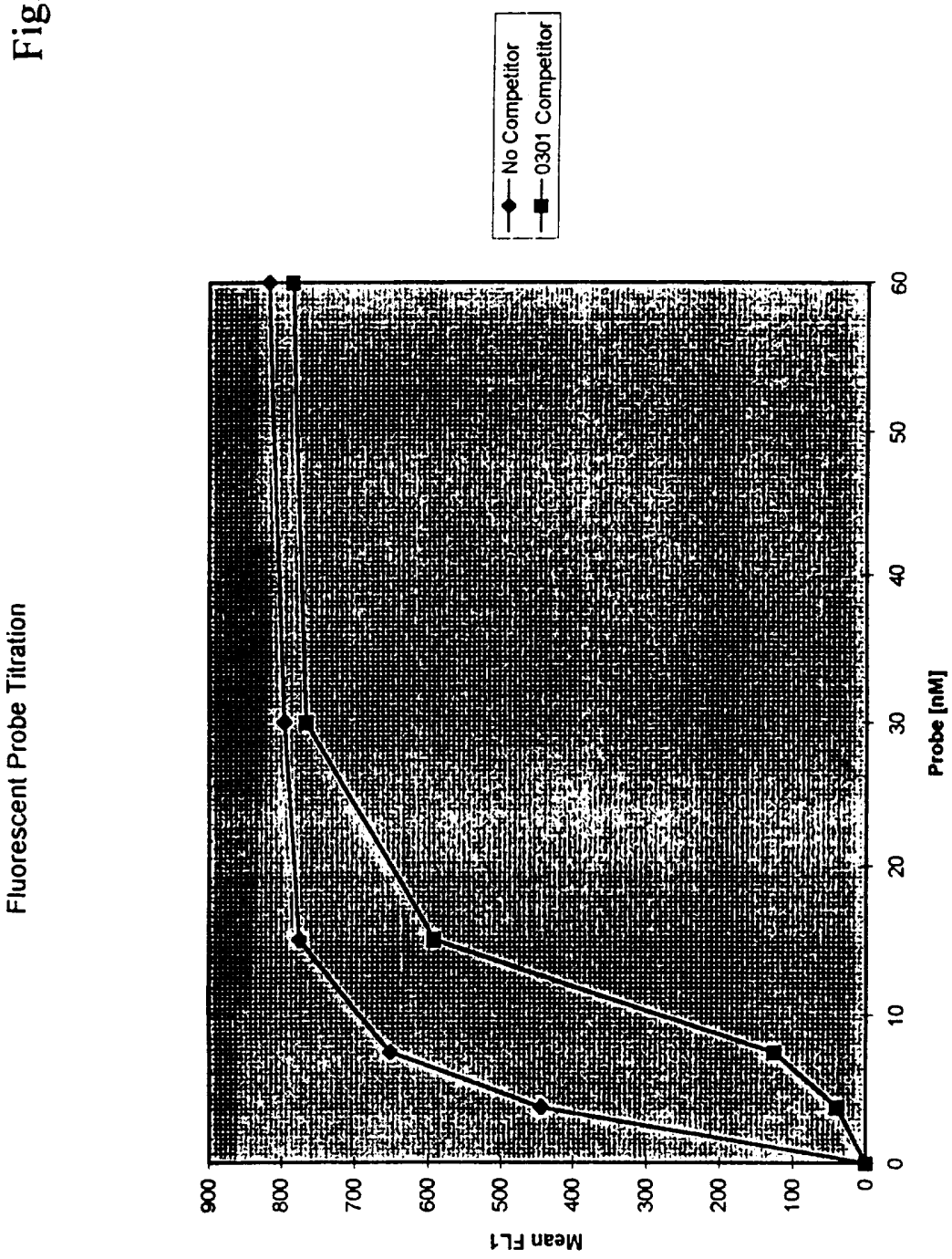

Fluorescent Probe Titration
No Competitor
0301 Competitor
Mean FL1
0
100
200
300
400
500
600
700
800
900
Probe [nM]
0
10
20
30
40
50
60

Fig. 47

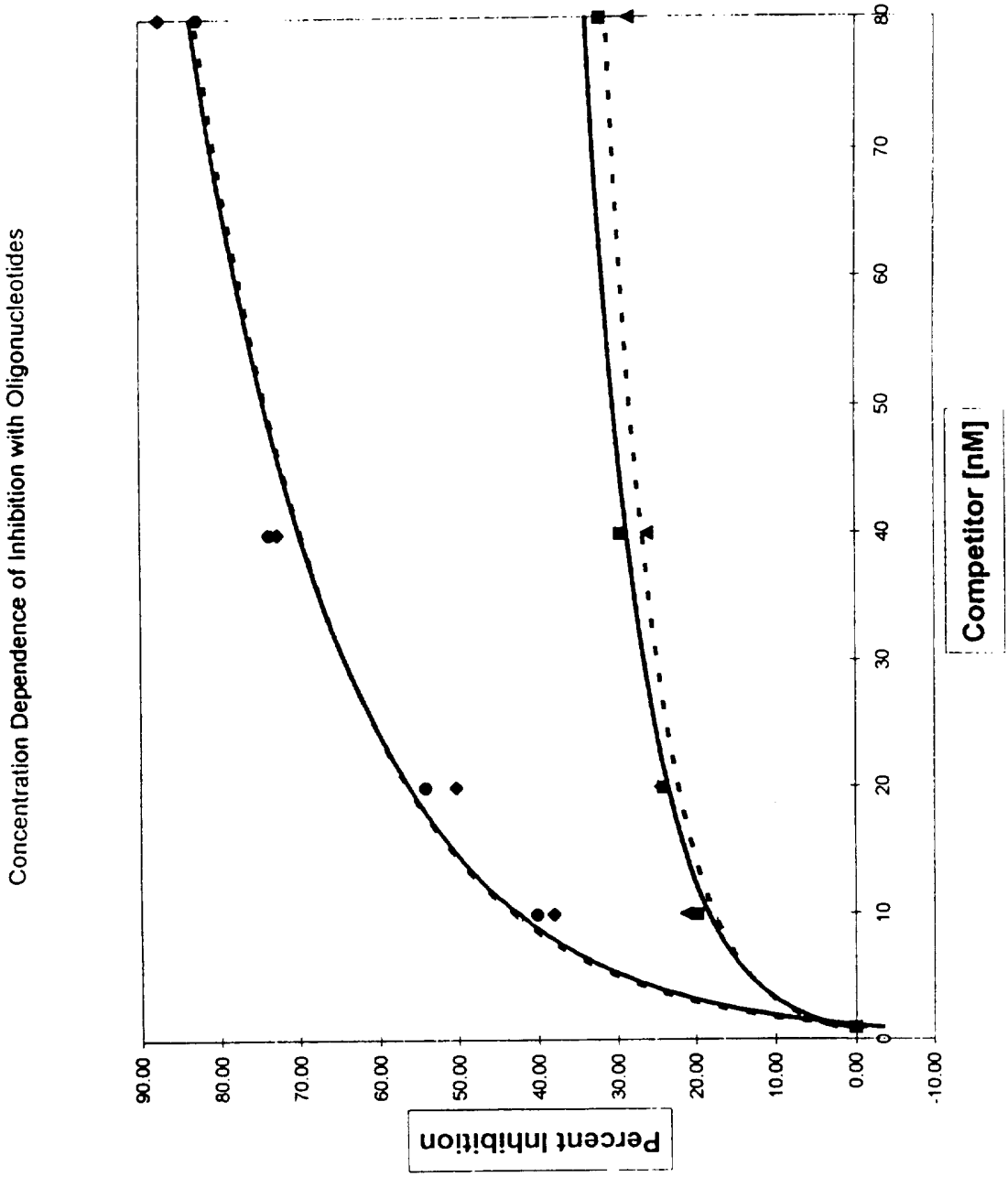
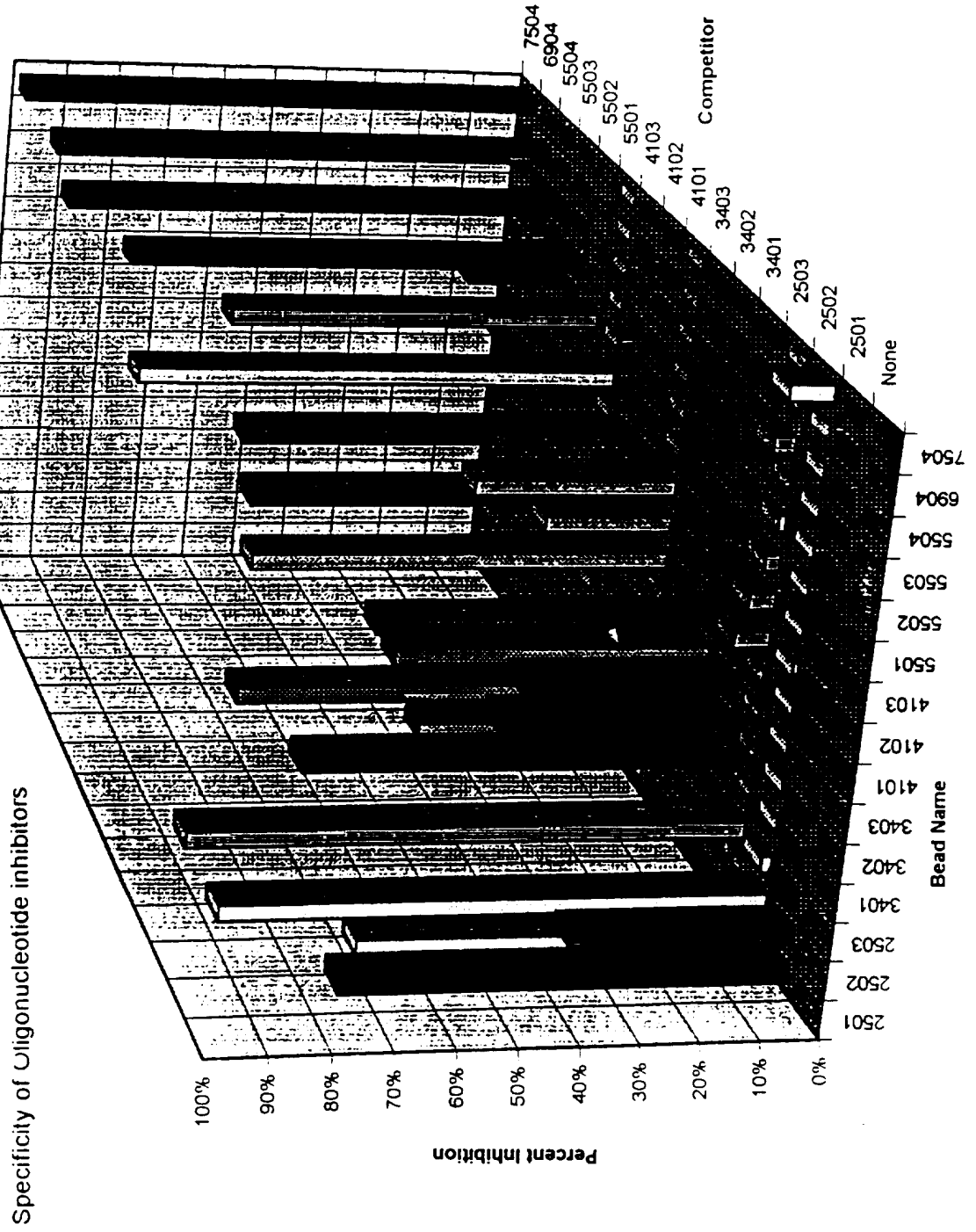

Concentration Dependence of Inhibition with Oligonucleotides
Percent Inhibition
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
0
10
20
30
40
50
60
70
80
Competitor [nM]

Fig. 48


Specificity of Oligonucleotide inhibitors
Percent Inhibition
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Competitor
7504
6904
5504
5503
5502
5501
4103
4102
4101
3403
3402
3401
2503
2502
2501
None
Bead Name
7504
6904
5504
5503
5502
5501
4103
4102
4101
3403
3402
3401
2503
2502
2501

Typing of Mock DQA1 Alleles

Fig. 49

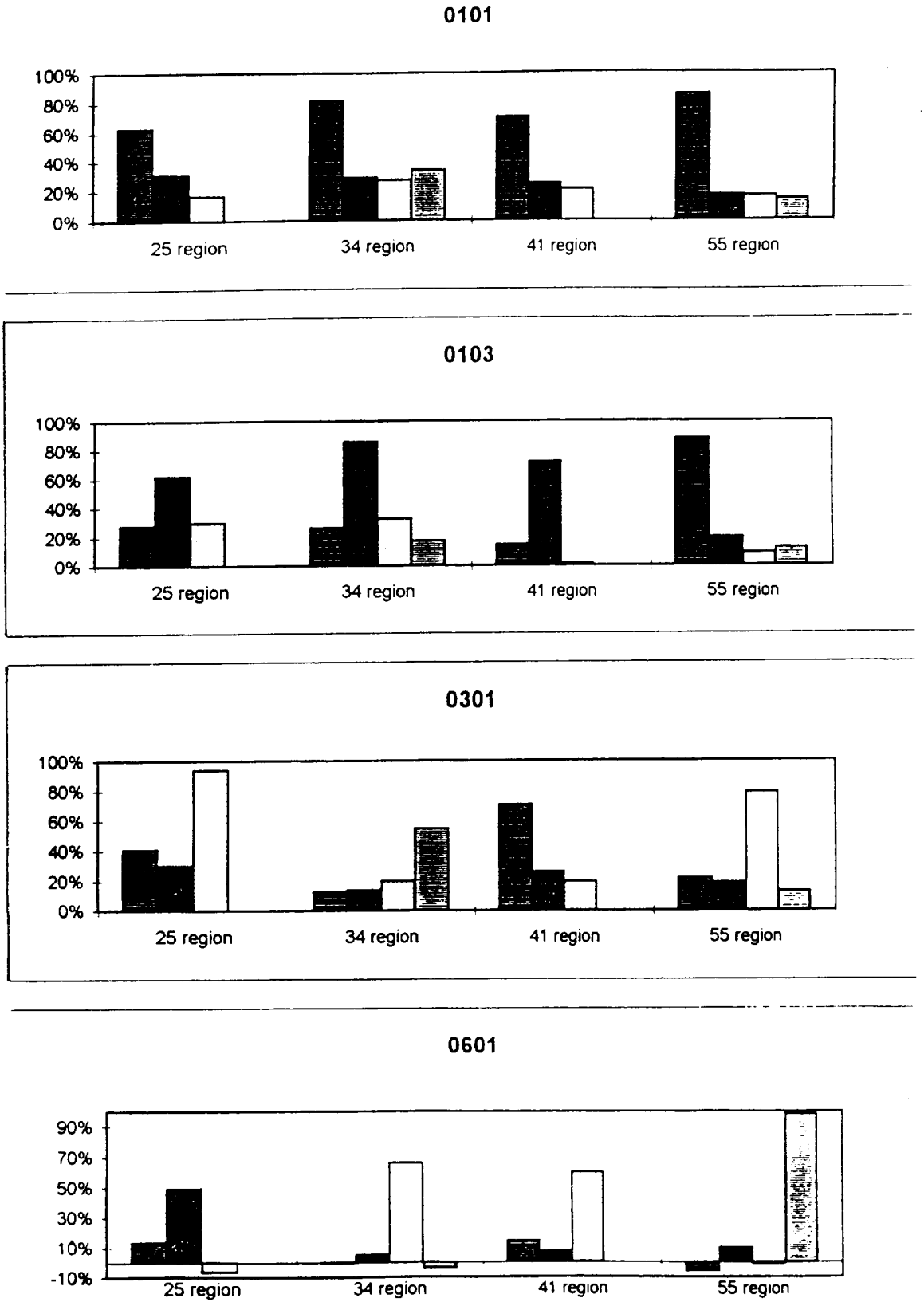


Fig. 50

Typing of DQA1 PCR Products

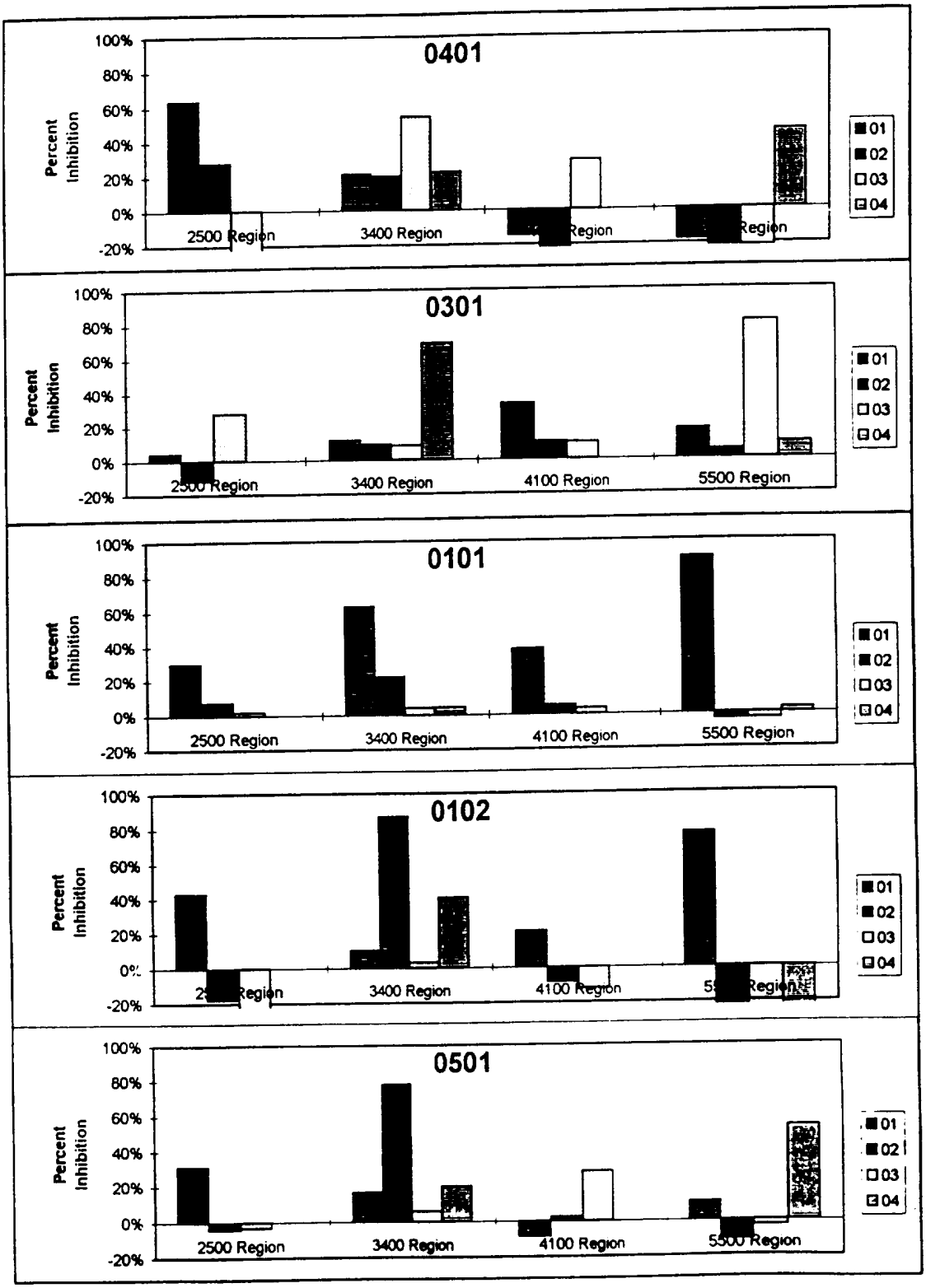


Fig. 51a

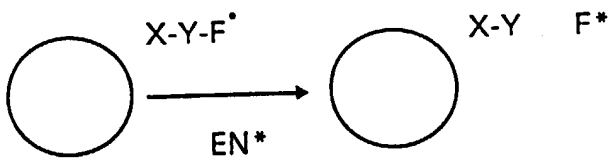


Fig. 51b

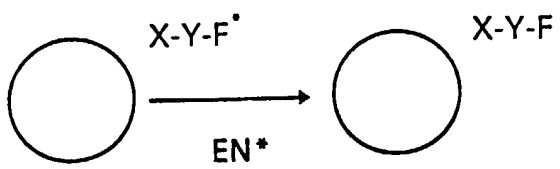


Fig. 51c

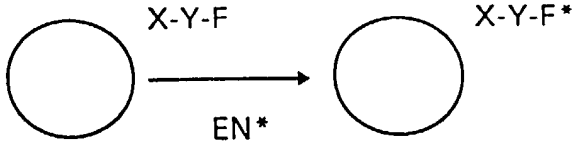


Fig. 51d

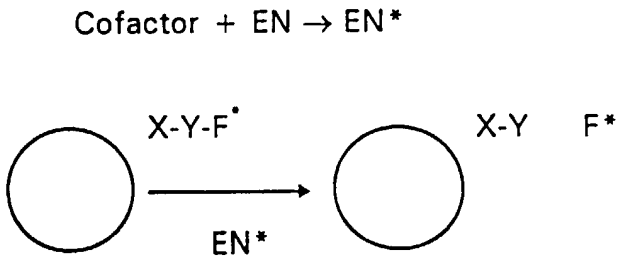


Fig. 51f

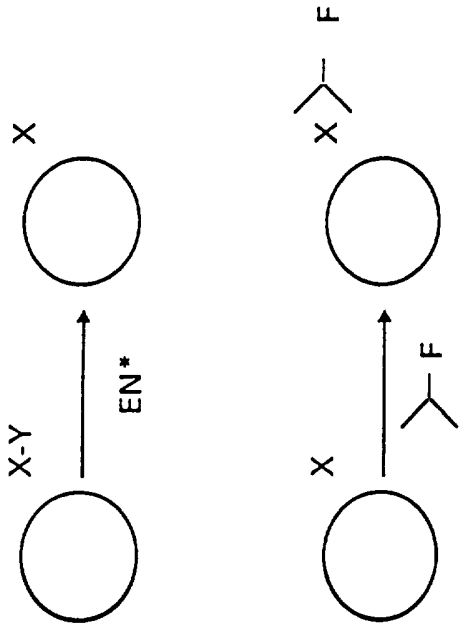


Fig. 51e

